Anatomical and Physiological Studies of the Recovery of Peripheral Nerve Function Following Repair With Freeze-thawed Skeletal Muscle Autografts.

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(Abstract)

The work presented in this thesis had two aims. The first was to show that freeze-thawed skeletal muscle autograft was as effective as other methods of peripheral nerve repair, in the animal model. The second was to gain some insight into the mechanisms of peripheral nerve regeneration, with particular emphasis on the recovery of muscle nerve supply, both motor and sensory.

Freeze-thawed skeletal muscle autografts were used to repair gaps of one centimetre in the rat sciatic nerve. This was compared to two standard methods of repair, autologous cable graft and direct epineurial suture. At various times after repair these methods were compared using anatomical and physiological indices of nerve recovery. Muscle function was assessed in the same rats using physiological methods. The sensory reinnervation of muscle was looked at in separate groups of rats using the horseradish peroxidase method of neuronal tracing. Muscle spindle reinnervation was looked at using histological methods.

The thesis concludes that freeze-thawed skeletal muscle autografts were as successful as autologous cable graft

in the repair of rat sciatic nerve but, as expected, were inferior to direct nerve suture. Since muscle grafts are easier to perform and are less disfiguring to the patient this technique should now be accepted as a method of peripheral nerve repair in the human when the defect cannot be repaired directly and a graft of some description is necessary.

DECLARATION

I, Lynn Marion Myles, declare that this thesis has been composed totally by myself and that the work reported here is my own work. Any technical assistance obtained has been acknowledged.

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CHAPTER 1

GENERAL INTRODUCTION

The repair of a completely transected nerve trunk constitutes a complicated and unique biological problem. The regenerating nerve fibres have to advance across a wound towards the distal nerve segment, a process which is influenced by a great number of factors many of them still unknown. Furthermore, wound healing is associated with local inflammatory-mediated changes in the microenvironment and the resultant hypoxic, hypercarbic, acidotic environment is far from what axons are adapted to within the endoneurial space. It is difficult enough when the severed ends are divided leaving a gap of only a few millimetres. It is almost impossible however, for regeneration to occur when there has been significant loss of neural tissue and the ends are separated by an extensive defect. One of the longstanding problems in the field of peripheral nerve repair is how best to repair a nerve which has been divided, with the introduction of a significant gap. It is generally accepted that the best method of nerve repair is by direct end to end suture without tension (Gutmann and Sanders, 1942; Terzis, Faibisoff and Williams, 1975) but this becomes more difficult with increasing loss of tissue and since there is no doubt that any degree of tension at the suture line has grave effects on the eventual outcome (Lundberg and Rydevik, 1973; Miyamoto,

1979; Miyamoto, Miyamoto & Arita, 1981), there are many occasions where the gap has to be bridged by a graft of some description. Many materials have been tried with varying degrees of success (Weiss, 1943; Marmor, 1964; Mackinnon, Hudson, Falk *et al*, 1984; Molander, Olsson, Engkvist *et al*, 1982). Hitherto, the only feasible method in these cases has been the use of a cable graft (ie. several strands of autologous donor nerve fashioned into a rope or "cable" which are then sutured or glued between the proximal and distal nerve stumps). The object of this thesis is to describe a new method of bridging these defects, the muscle graft, and to show that it is at least as effective as other methods of repair as measured by anatomical, physiological and clinical indices, with particular emphasis on the recovery of muscle nerve supply, both motor and sensory.

1:1 What Happens When a Nerve is Severed?

This section describes the changes taking place in a nerve when its fibres are damaged. This has been investigated largely in animal subjects which only serve to highlight the variability of the response according to species, age and severity of the injury. It is always dangerous to extrapolate from animal to human, nevertheless the reaction is broadly similar in many respects from species to species often varying only in timing or severity. Bearing these points in mind the following account should be taken as a general outline of the events occurring in mammalian nerves following

severance of the axon.

1:2 Degeneration

When a nerve is severed the stumps immediately retract and undergo a series of changes leading to the degeneration of the nerve fibres. When this occurs in the distal stump it is known as Wallerian degeneration. A similar, retrograde degeneration occurs in the proximal stump to a varying degree. In addition, reactive changes can be seen in the cell bodies of these neurones in the dorsal root ganglia (DRG) and the anterior horn of the spinal cord.

1:2:1 Wallerian Degeneration

After injury, axons in the distal stump start to degenerate. After a crush injury to rat dorsal root, axonal degeneration was reported to have been almost complete by 48 hours, (Nathaniel and Pease, 1963a). However after total severance of the axon these changes were delayed. After 48 hours in cut rabbit sural nerve the axons had only just started to show signs of degeneration (shrunken and granular cytoplasm) with maximum degeneration occurring at 72 hours (Barton, 1962). After a crush injury, myelin sheath degeneration was well underway by 96 hours and extensive disintegration could be seen at one week (Nathaniel and Pease, 1963a). After total severance extensive myelin degeneration was not apparent until two weeks after injury (Barton, 1962). In both cases however, myelin

debris was found for over three months, in vacuoles and phagosomes, as well as lying free in the cytoplasm of the Schwann cell. The basement membrane formed a continuous sheath surrounding the Schwann cell and nerve fibre. These tubes ("endoneurial tubes") were continuous across the nodes of Ranvier (Thomas, 1964a). They persisted during Wallerian degeneration although with time they tended to collapse, producing marked infolding of the basement membrane (Nathaniel and Pease, 1963c; Thomas, 1964b). This effect was compounded by the increasing production of endoneurial collagen fibrils associated with an increase in the number of endoneurial fibroblasts, which caused the endoneurial tubes to contract still further (Barton, 1962; Holmes and Young, 1942; Thomas, 1964a,b). The production of endoneurial collagen was linear up to 200 days and was greatest nearest the point of injury (Abercrombie and Wilson, 1946). The production of endoneurial collagen seemed to be a constant feature, up to 100 days, whether or not reinnervation took place (Abercrombie and Johnson, 1946).

The Schwann cell reaction is one of proliferation. By three to four days the Schwann cells had taken on a reactive appearance, with increased ribosomal clumping in the cytoplasm, coming to resemble active fibroblasts (Barton, 1962; Nathaniel and Pease, 1963a; Thomas, 1966). These reactive Schwann cells became mobile and started to line up, forming solid cords known as Bungner's bands, within the basement membrane tubes (Abercrombie and

Johnson, 1942; Barton, 1962; Nathaniel and Pease, 1963b). In the severed nerve, Schwann cells migrated from the cut end of the distal stump to form a glioma, along with perineural cells and fibroblasts (Thomas, 1966). This outgrowth could bridge the gap between the severed ends if it was not too extensive or if the ends had been surgically apposed (Sunderland, 1978). Schwann cell migration began to increase four days after injury, reaching a maximum at 19 - 25 days. There was a 40-fold increase in Schwann cell migratory activity in tissue culture at this time. If the nerve was not repaired the activity reached a plateau at this level until approximately 60 days when the rate of migration started to fall. However at one year there was still considerable migratory activity (five to seven times as high as day four, Abercrombie and Johnson, 1942).

The role of the Schwann cell in the production of nerve growth and chemotactic factors is only now being investigated. Dividing and migrating Schwann cells have been shown to express increased numbers of nerve growth factor (NGF) receptors. Seven days after injury NGF receptors had increased 50-fold distal to the lesion (Taniuchi, Clark and Johnson, 1986). NGF was not normally synthesised within the sciatic nerve but local NGF synthesis and levels of messenger ribonucleic acid (mRNA), specific for NGF, increased 15-fold distal to the transection site. This increase started at six hours and was still present at two weeks (Heumann, Korsching, Bandtlow *et al*, 1987).

Laminin is an extracellular, non-collagenous, matrix glycoprotein which is present in all basement membranes. It can be produced by epithelial cells in culture and has been found to promote the adhesion of cells to a substrate *in vitro* (Bigmami, Chi and Dahl, 1984). Staining with immunofluorescent laminin antisera showed that laminin was confined to the endoneurium in the normal nerve. Similar staining showed that by day 11 after injury, proliferating Schwann cells in the bands of Bungner had become laminin positive (Bignami, Chi and Dahl, 1984). The exact role played by the Schwann cell is yet to be elucidated but it seems to be involved in axon guidance as well as axon ensheathment. The role of the Schwann cell in Schwann cell-axon interaction and axon outgrowth will be discussed further, later in this chapter.

Nerve grafts contain endoneurial tubes and a population of live Schwann cells (Aguayo, 1981). They undergo degeneration in the same way as the distal stump (Holmes, 1947; Sanders and Young, 1942).

1:2:2 Degeneration in the Proximal Stump

The retrograde reaction is essentially the same as that occurring below the site of the lesion. Although in most textbooks of physiology it is still stated that retrograde degeneration is confined to the preceding node of Ranvier, it is now known that degeneration extends centrally for a distance which varies according to the severity of the injury. After a crush injury, retrograde

degeneration is confined to the adjacent few millimetres of the fibre (Greenman, 1913; Lubinska, 1959, 1961). However, in severe injuries, the effects may extend proximally for several centimetres and are usually associated with swelling and oedema of the nerve trunk (Ducker, 1972). In the longer term it has been shown that these changes in the proximal stump are associated with a decrease in fibre diameter, which is reflected in a reduced conduction velocity, (80% of normal at 50 days, Cragg and Thomas, 1961). These variables return to normal if contact with the periphery is achieved.

1:2:3 Reactive Changes in the Neuronal Cell Bodies

Dividing an axon may bring about a series of changes in the cell body which are known as chromatolysis. This does not invariably happen to every cell and the factors which bring about chromatolysis are poorly understood. The initial changes involve the structure, biochemistry and function of the cell and constitute a reactive phase from which the cell may or may not recover. These changes and the degree of cell loss after peripheral nerve repair will be discussed more fully in chapter 4.

1:3 Regeneration

1:3:1 Each proximal axon stump sends out several unmyelinated axon sprouts (Bray and Aguayo, 1974). The first axon processes were seen in the distal nerve at seven days after a crush injury and at fourteen days after severance and epineurial suture (Barton, 1962).

Axons were seen to grow for short distances unsupported by other cells possibly attracted by soluble factors diffusing from the distal stump (Hall, 1986; Keynes, 1987; Lundborg and Hansson, 1981; Lundborg, Dahlin, Danielsen *et al*, 1982a,b). However, organised growth failed after a few millimetres if the axon was not presented with either a degenerate distal stump (Nadime, Anderson and Turmaine, 1990) or, in culture, with the products of the distal stump (Scaravilli, 1984; Ard, Bunge and Bunge, 1985). In the distal stump, axons were seen to grow into the Schwann cell-filled endoneurial tubes (Holmes and Young, 1942). In culture they also grew along Schwann cell extracellular matrix alone (Ard, Bunge and Bunge, 1985).

1:3:2 The Role of the Basement Membrane

The basement membrane (BM) is an extracellular scaffold common to many tissues. Its components include type IV collagen, laminin, heparan sulphate glycoprotein, entactin and fibronectin (Mohan and Spiro, 1986). It is positioned between parenchymal cells and connective tissue. Cells attach to one surface and connective tissue to the other. The BM defines the spatial relationships between the cells and the connective tissue elements in a tissue. Replacement of cells occurs in an orderly way along the framework of the BM in many tissues. If the BM is destroyed, healing in most tissues results in the formation of scar tissue (Vracko and Benditt, 1972; Vracko, 1974). The peripheral nerve is no

exception, the BM of the endoneurial tube playing a significant role in nerve regeneration.

1:3:3 BM - Schwann Cell - Axon Interactions

Schwann cells are derived from ectoderm, coming from the neural crest during development (Gray's Anatomy, 36th edn. 1980). The epithelial nature of the Schwann cell is expressed even within the peripheral nerve. The Schwann cell positions itself between the nerve cell and connective tissue elements and, as in all epithelia, deposits a BM on its external aspect (Gamble, 1964). Thus, each axon-Schwann cell unit is a fragment of epithelium with axon and Schwann cell closely packed without an intervening extracellular matrix but with each unit surrounded by a BM (Bunge, 1987). It has been shown that the Schwann cell produced components of the BM, that deposition of this lamina was regulated by contact between Schwann cell and axon (Bunge, Williams, Wood *et al*, 1980), and that Schwann cell function was retarded when BM failed to form (Bunge and Bunge, 1983). However, the BM once formed did not require the presence of axons for its persistence (Bunge, Williams and Wood, 1982).

Normal Schwann cell function, including axon myelination, requires the simultaneous presence of collagen. It has been suggested that a substratum of collagen was required to allow the Schwann cell to spread longitudinally along the axon (Bunge and Bunge, 1978).

It would seem from this that in order to function fully

the Schwann cell requires to develop a polarisation, one side of the cell being in contact with axon while the other is in contact with collagen. It is this latter end of the cell which eventually secretes BM components, to form a new BM, away from the axonal pole. In the degenerate distal stump of a regenerating peripheral nerve, Schwann cells polarise along the existing BM framework (containing type IV collagen) of the endoneurial tubes to form Bungner's bands (Nathaniel and Pease, 1963b). A new BM is formed by the Schwann cell, in contact with the old BM (Nathaniel and Pease, 1963c), while the opposite pole of the cell enfolds the regenerating axon. In these circumstances myelination can proceed (Brockes, Fryxell and Lemke, 1981).

1:4 The Formation of the Myelin Sheath

The bare axon sprout was always in advance of the changes which took place to restore the original structure of the nerve fibre. The Schwann cells aligned themselves along the length of the axon and formed a mesaxon which then encircled the regenerating axon by repeated turns to form a multilamellated sheath in the same way as occurs in developing nerve fibres (Friede and Samorajski, 1968). Myelination has been reported to occur when the axon sprout reaches a critical diameter of the order of 1.5-2.0 μm (Quilliam, 1956). It then proceeded centrifugally down the fibre (Sunderland, 1978). Remyelination has been found to commence as early as the seventh day after a crush injury (Nathaniel and Pease,

1963b) and also after direct nerve suture (Hudson, Morris and Weddell, 1970). However, remyelination may not have reached normal levels even at one year after injury (Barton, 1962). The extent of myelination has an important effect on conduction velocity and therefore the function of the nerve fibre. This will be discussed more fully in chapter 3.

1:5 The Problems of Peripheral Nerve Repair

Improvements in the results of peripheral nerve repair over the last 50 years have been due in part to:

1) better quality optics and surgical instruments allowing finer, more exact surgical apposition of the traumatised nerve ends
2) improved suture materials and different suture techniques
3) more effective ways of controlling wound infection including the introduction of better surgical technique and the use of antibiotics
4) a clearer understanding of the structure of the peripheral nerve and, with this, the realisation that the problem is much more complex than was originally thought.

The results of direct suture under optimal conditions can be good but the results of the repair of large defects are still disappointing.

1:5:1 Nerve Grafting

The problem of nerve grafting is not a new one. It

includes all procedures in which a bridge of nervous tissue is inserted between the cut ends of a nerve. Nerve grafts may be **autografts** (pieces of nerve from the same individual), **allografts** (pieces of nerve from another individual of the same species), or **xenografts** (pieces of nerve from an individual of a different species). Grafts of all three types may be used fresh or after such treatment as fixation, freezing and thawing or irradiation, when they are known as bioprostheses.

The pioneering work of Philipeaux and Vulpian in 1870 showed for the first time that a grafted nerve could be made to conduct new nerve fibres across a gap. This was followed in 1878 by the first recorded nerve allograft in a human patient. This was performed by Albert, (ref.1885), who transplanted a nerve from an amputated limb to bridge a three centimetre defect after the removal of a sarcoma of the median nerve.

Experimental work with allografts in animals has not been entirely satisfactory. In some cases the allograft has united with the host nerve and allowed initial penetration by axon sprouts, only to become replaced by fibrous tissue before axons had bridged the defect (Verga, 1918; Eden, 1919; Huber, 1920). However, Gutmann and Sanders (1942) and Sanders and Young (1942) showed that allografts, like autografts, became united with the host nerve but thereafter their behaviour differed. The most striking feature of the reaction was its extreme variability. However they were able to conduct new fibres across a gap of two centimetres in sufficient

numbers to produce a return of motor function and sensiblity, although this was inferior to that of autografts. The most striking histological feature of the allograft was its invasion by lymphocytes. In some cases this reaction completely destroyed the graft.

Histological investigations of the inflammatory changes that take place within nerve allografts have shown that the response of the host tissue to such grafts resembles that seen in skin allografts (Medawar, 1944,1945). The extent of the host reaction depended on the amount of antigenic material present, ie. on the length and thickness of the graft. One of the main features distinguishing human and animal allograft experiments has been the size of transplants used, the human grafts being much larger. This is one of the reasons why human allografts have done so badly (Seddon and Holmes, 1944). The level of the immune response can be reduced by matching for HLA antigens (Mackinnon, Hudson, Falk *et al*, 1982). Procedures devised in an attempt to reduce the host reaction to unmatched nerve allografts have added little to the success of the technique (Gutmann and Sanders, 1942; Mackinnon, Hudson, Falk *et al*, 1984).

Xenografts do not make proper unions with the host nerve and do not degenerate (Medical Research Council special report series, No.282, 1954). They set up a violent host reaction, are quickly destroyed and have no part to play in peripheral nerve repair.

This leaves the autograft as the preferred method of nerve graft.

Sanders and Young (1942) showed that small, thin autografts in rabbit lateral popliteal nerve were able to survive, to degenerate and to conduct new nerve fibres across the gap in the nerve at a rate of approximately 2 mm/day. This rate was only a little slower than the 3.5 mm/day at which they grew into the distal stump after direct end to end repair. Regeneration of axons through the graft was followed by the return of motor and sensory function, although never to normal levels.

In humans it is very rare to be able to use a full thickness nerve trunk graft, since this necessarily involves sacrificing a large motor or mixed sensory and motor nerve. The resultant loss of function in the territory of that nerve would be unacceptable, although occasionally, when a limb cannot be saved and has to be amputated, the large nerves may be harvested from this, to produce full thickness grafts for repair of other nerves in that patient.

Autologous cable grafting refers to a technique which attempts to simulate a nerve trunk graft where this is not an option. Several small strands of autologous nerve (usually sensory) are fashioned into a thicker rope or "cable" which is sutured or glued between the proximal and distal stumps of the injured nerve. Smaller, sensory nerves can be sacrificed with little loss of function although several unsightly scars may result (Seddon, 1975). It is important to note that the results of full thickness autografts in the human are superior to those obtained from repair by cable graft (Bjorkesten, 1948;

Sunderland, 1978) and that trunk autografts, even when they consist of nerve segments of considerable length and thickness, offer reasonable prospect of success in the repair of human nerve lesions (Holmes, 1947; Sunderland, 1978). They are however always inferior to direct nerve suture if this can be performed under ideal conditions. (Gutmann and Sanders, 1942). The reasons for this may be summarised as follows:

1) **The Suture Line**

There are two sites of coaptation to transgress instead of one as in direct end to end nerve repair. Problems arising at the site of coaptation irrespective of the method used include

a) Delay

The approximate delay at the site of coaptation in rabbit nerve was 7.27 days after plasma suture with the rate of axon growth after this being 3.42 mm/day. (Gutmann, Gutmann, Medawar *et al*, 1942). In rat nerve grafts it took 28 days for all axons to pass the proximal suture line (Miyamoto, Miyamoto and Arita, 1981). This delay is much more marked in the early months after repair with regeneration being much more advanced at this time after direct epineurial suture (Kline, Hudson and Bratton, 1981). Obviously there is a degree of hold-up of axon growth at the site of coaptation but some of this delay must be due to the time taken for regeneration from the point of initial growth to the end of the proximal stump. This length will vary, depending on the degree of injury,

as discussed earlier in this chapter.

b) Tension

Nerves are elastic structures and when they are cut the ends tend to retract. A degree of force is therefore required to reunite the cut ends. This force increases as the gap between the ends increases (Millesi and Meissl, 1981). Lundborg and Rydevik (1973) using an intravital microscope to observe the circulatory changes occurring in rabbit tibial nerve during stretch, showed that circulatory disturbance in the proximal stump began to appear at only 8% stretch and that the microcirculation was completely arrested at 15% stretch. Miyamoto (1979) showed similar and irreversible results in dog nerve beginning at 5% stretch. (Figure 1)

The consequences of such intraneural ischaemia are disastrous with a marked reduction in the number of axons being found in the distal stump and a zone of collagen scar tissue being produced at the suture line (Millesi and Meissl, 1981). It has been shown that there is a direct relationship between the amount of connective tissue proliferation and the degree of tension at the suture line (Terzis, Faibisoff and Williams, 1975). As the degree of tension required to unite the ends of a nerve increases, the effects of tension may begin to outweigh the extra delay at a second suture line and a graft may become the best method of repair. This assumes that each graft suture line is made under ideal conditions (no tension nor infection, good blood supply and good surgical technique).

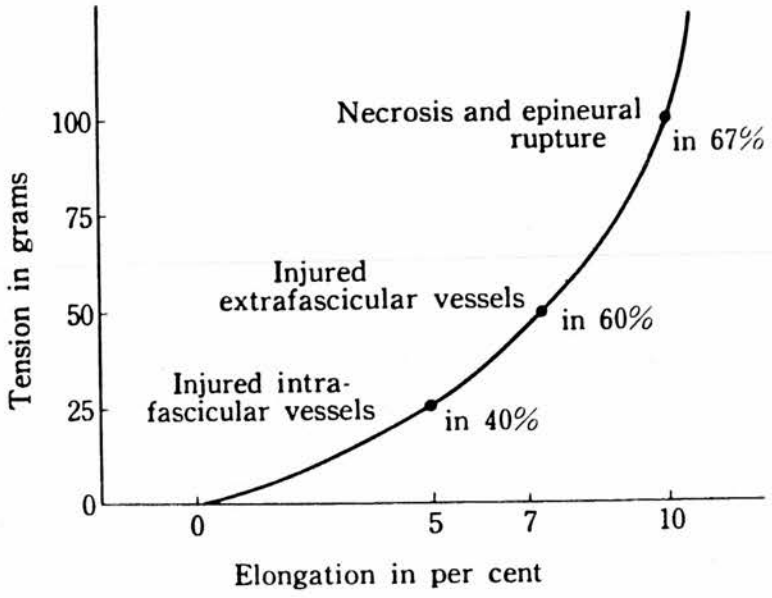


Figure 1: Effects of Tension at the Suture Line.
Diagram indicates the relationship between tension at the suture line and the state of circulatory disturbance on the proximal side of the nerve trunk. (From Miyamoto, Y. Experimental Studies on the Repair of Peripheral Nerves. Hiroshima Journal of Medical Science, *28*, 87, 1979, with permission)

c) Loss of Axons into Connective Tissue

Hudson, Morris, Weddell *et al*, (1972) concluded that the crucial factor in determining the success of a nerve graft was the relative orientation of the fascicles of the graft compared to those of the proximal and distal stumps. A major loss of efficiency was due to loss of

axons at the suture lines because of their ingrowth into extrafascicular connective tissue. Millesi (1981) has stated that up to 10-15% of axon sprouts may be lost into the connective tissue surrounding nerve grafts or lying between nerve fascicles. The problem is made worse when a cable graft is used since the space between individual nerve strands is rapidly filled with a connective tissue matrix through which axons can be seen to grow (Figure 2).

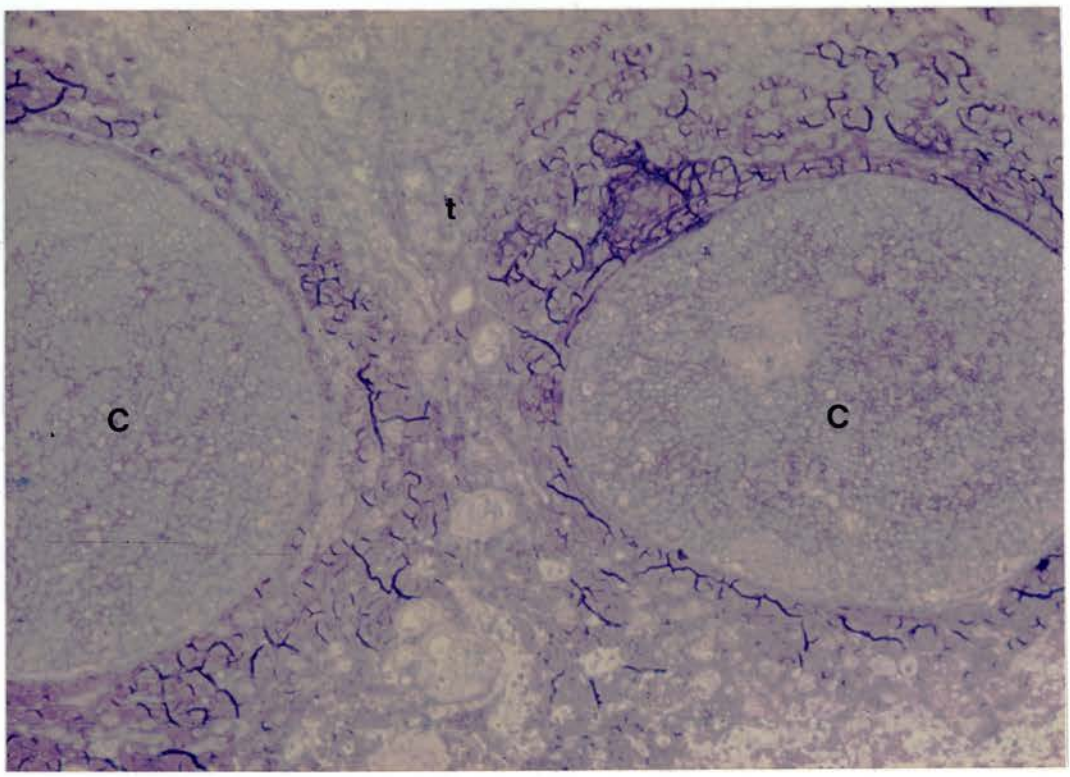


Figure 2: A semithin, plastic embedded, Toluidine Blue stained section of a cable graft. (50 days, x175)
c = cable, t = connective tissue.

d) Foreign Body Reaction to Suture Materials

A foreign body reaction to suture materials can occur at the suture line as it can anywhere in the body. This leads to chronic inflammation and its inevitable companion, fibrosis (Anderson, 1980). This problem occurs less frequently now that relatively inert suture material is routinely used. 10/0 polypropylene monofilament or polyamide is now favoured for this reason.

Fibrin glue methods of coaptation also produce favourable results when compared to epineurial suture (Young and Medawar, 1940; Gutmann, Gutmann, Medawar *et al*, 1942; Becker, Guering and Graff, 1985), and excite little foreign body reaction if used properly.

2) The Funicular Pattern of the Graft

The funicular pattern of the graft must differ from that of the donor nerve since the funicular pattern is never exactly the same in any two nerves (O'Connell, 1936; Sunderland and Ray, 1947).

The nerves commonly used to furnish grafts are small sensory nerves which can easily be removed with little loss of function eg. sural nerve. These nerves contain small diameter fibres of the range 1-15μm. Large motor and sensory fibres from muscle have diameters of 15-20μm (Gray's Anatomy 36th Edition, 1980). The graft will therefore furnish endoneurial tubes of inappropriately small size. This may be one of the reasons why large diameter fibres are seldom seen after this type of repair

(Hammond and Hinsey, 1945).

3) **The Funicular Pattern of the Host Nerve**

Only relatively large gaps are repaired with grafts, therefore a significant amount of neural tissue will have been lost in these cases. The funicular pattern is constant for a maximum of only one centimetre in human nerves (O'Connell, 1936; Sunderland and Ray, 1947) and so a very different funicular pattern may be found in the proximal and distal nerve faces, making it difficult to match fascicles. Even if fascicles can be matched there is evidence of microfascicular plexus formation in human nerves (O'Connell, 1936; Sunderland and Ray, 1947). Cable grafts offer an advantage here over full thickness grafts. Each nerve strand can often be matched to an individual fascicle or group of fascicles but, the more strands which are used, the more connective tissue and suture material is contained within the graft and the more axons subsequently may be lost (as described above). Also, individual fascicles have to be dissected free with some disruption of the proximal stump and loss of some of their blood supply (Sunderland, 1979).

4) **Surrounding Trauma**

Large gaps in nerves are often associated with extensive surrounding tissue trauma and loss, *e.g.* gunshot wounds. Vascular injuries causing ischaemia and infection are of particular significance. Both will severely compromise the capacity of a nerve to regenerate (Medical

Research Council, 1954).

In addition to these considerations, the technique of cable grafting is lengthy and technically demanding especially if funicular grafting is undertaken and considerable microsurgical expertise is required. Furthermore a normal, healthy cutaneous nerve has to be sacrificed in order to furnish material for the graft, leaving the patient with one or more unsightly scars and a potential area of anaesthesia in the area supplied by that nerve.

The perfect graft material should fulfill the following criteria:

i) it should be easily available and easily tailored to an appropriate size

ii) it should not cause an immune reaction in the host

iii) it should contain large diameter tubes of sufficient diameter to be able to accommodate the largest nerve fibre

iv) it should have a chemical composition similar to the endoneurial tubes of degenerating nerve

v) it should offer the most direct route to the distal stump, be able to support actively growing axon sprouts and allow full maturation of nerve fibres.

Glasby *et al* (1986a,b,c,d; 1988; 1990) discovered that freeze-thawed, co-axially aligned, autologous skeletal muscle fulfilled these criteria and was also capable of supporting nerve regeneration.

1:6 The Muscle Graft

The idea that perhaps nerves could regenerate through muscle is not a new one. As long ago as 1865, Waldeyer noted that the BM of muscle was an important guiding factor for regenerating myocytes and remarked on the similarity between this and the role of the endoneurial tube in guiding axons during nerve regeneration. It has been confirmed that endomysial tubes play an important part in guiding new muscle fibres during regeneration (Le Gros Clarke, 1946). The BM of muscle remained unchanged during degeneration (Allbrook, 1962; Vracko and Benditt, 1972; Gulati, Reddi and Zalewski, 1983) and was shown to contain laminin, type IV collagen and heparan sulphate glycoprotein (Kuhl, Timpl and Von der Mark, 1982; Gulati, Reddi and Zalewski, 1983; Sanes and Cheney, 1982). Vracko (1974) described at least three sets of BM tubes in degenerate skeletal muscle (Figure 3), belonging to muscle fibres, blood vessels and nerves contained within the muscle. The approximate size of the muscle BM tubes is 40-100 μm (Ide, Tohyama, Yokota *et al*, 1983) which is large enough to accommodate the largest nerve fibre.

The regenerating myoblasts were found inside the BM tubes (Allbrook, 1962; Vracko, 1972) in the same way as regenerating neurites were found within endoneurial tubes (Holmes and Young, 1942; Ide, Tohyama, Yokota *et al*, 1983). They produced a new BM within the existing BM scaffold in the same way as Schwann cells deposited new BM within endoneurial tubes (Nathaniel and Pease, 1963c).

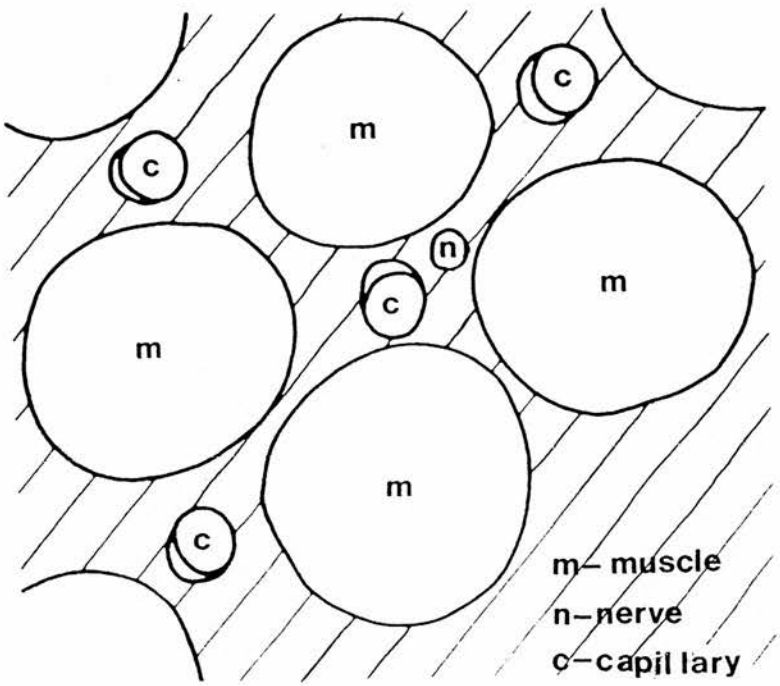


Figure 3: Diagramatic representation of the basement membrane tubes of degenerate skeletal muscle.

Freeze-thawed nerve grafts contained no live Schwann cells but only a BM matrix and cellular debris which was quickly removed. Axon sprouts could regenerate along the inner surface of this BM. By day 5-7 they were found to be accompanied by Schwann cells which had migrated from the proximal stump (Ide, Tohyama, Yokota *et al*, 1983). The BM of other tissues will also support the outgrowth of neurones in culture (Davis, Blaker, Engvall *et al*, 1987).

When Schwann cell outgrowth from the proximal stump was

inhibited by an injection of mitomycin-C, an antimitotic agent, axon growth into a freeze-thawed nerve graft was markedly reduced and no myelination occurred (Hall, 1986).

The earliest reference to the use of muscle in nerve repair was by Causey (1955) in a Hunterian lecture at the Royal College of Surgeons of England. He used autogenous, fresh skeletal muscle transposed on its vascular pedicle as an interposed graft in the rabbit sciatic nerve. He compared his results with interposed free peroneal nerve autografts. He found that there were fewer fibres seen in the muscle grafts and that maturation was retarded. This was presumably seen as an adverse indicator and the work was not followed up.

Ide (1984) produced freeze-thawed muscle grafts from mouse biceps femoris muscle and sutured them to the proximal stumps of cut sciatic nerves. He showed that, at 2-5 days, the dead muscle cells were phagocytosed by macrophages leaving the BM intact. The first regenerating axon sprouts were seen in the proximal part of the graft by 7 days. Schwann cells were noted between 7 and 10 days, presumably having migrated from the proximal stump, there being no contact with the distal stump. By day 17, myelination had begun. Perineurial cells were evident from 25 days when the beginnings of compartmentation were seen. Similar results were found in the mouse, using either ethanol and formaldehyde, or distilled water to degenerate the muscle graft. The

first axons were seen at 4 days. More than 90% of axons were contained within the BM tubes. No growth was found when the muscle was preserved intact using glutaraldehyde or in live innervated muscle (Keynes, Hopkins and Huang, 1984).

In these respects a degenerate muscle graft seems to function in the same way as a freeze-thawed nerve graft and so it may be presumed that Schwann cell migration into the graft is a prerequisite for full axonal ingrowth and maturation, as it is in the freeze-thawed nerve graft.

Using a chemical method for evacuating the myoplasm Fawcett and Keynes (1986), showed that treated muscle grafts inserted into 4cm gaps in rabbit sciatic nerve were as successful, in terms of the number of myelinated axons found distal to the graft, as live nerve grafts of similar size. They also went on to measure the conduction velocities of these fibres but these results cannot be relied upon, since their fastest conduction velocity in normal nerve was found to be only 31.6 m/sec, which is very low.

Glasby, Gschmeissner, Hitchcock *et al*, (1986a), using freeze-thawed muscle grafts showed that they would support axonal regeneration, with good growth of myelinated axons into the distal stump and with recovery of function becoming apparent at 30-50 days. This was followed up with electrophysiological evidence of recovery at 51 days (Glasby, Gschmeissner, Hitchcock *et al*, 1986b). It was then shown that recovery of axon

numbers was slower in muscle grafts than in fresh nerve grafts of equal diameter at 50 days. However they had reached the same level by 100 days (Glasby, Gschmeissner, Hitchcock *et al*, 1986c). This is in keeping with the findings that axon penetration into freeze-thawed nerve grafts is slightly delayed when compared to fresh nerve grafts (Anderson, Mitchell, Mayor *et al*, 1983). This is presumably due to the time taken for debris to be cleared from BM tubes by macrophages and for Schwann cell migration to take place.

The findings described in the previous paragraph were confirmed in marmosets by Glasby, Gschmeissner, Huang *et al* (1986d). They showed that although the number of fibres in the muscle grafts was not significantly different from normal controls, the degree of myelination in the graft had not reached control values at six months. Myelination was slightly delayed when compared to the distal stump.

Recovery of the compound action potential and chronaxie values for muscle-grafted rat nerve were shown to be not significantly different from normal at 300 days although conduction velocities were markedly decreased, with only 50% of experimental animals having velocities greater than 25 m/sec. However this was a relatively crude analysis of conduction velocity, measuring only the velocities of one group of fibres within the nerve (Glasby, Gattuso and Huang, 1988).

Recovery of neuromuscular transmission was demonstrated after repair by muscle graft, both histologically and

physiologically (Gattuso, Davies and Glasby *et al*, 1988). It is important that the muscle graft is compared to other grafting techniques. When morphometric indices of recovery were compared in the femoral nerve of sheep, repaired with autologous freeze-thawed muscle graft and with three-strand cable graft, there were no significant differences in the number of axons distal to each of the grafts at ten months (Glasby, Gilmour, Gschmeissner *et al*, 1990).

Preliminary studies in humans have been encouraging. Autologous freeze-thawed muscle graft has been used to repair severed digital nerves. Sensory recovery to MRC grade S3+ (Zachary, 1954) was found after 11 months, which represents an excellent degree of recovery. (Norris, Glasby, Gattuso *et al*, 1988).

The object of this thesis is to expand on this work to show whether the muscle graft is as good as recognised methods of nerve repair in terms of axon numbers, fibre maturation and electrophysiological measurements of nerve and muscle function function. In particular, the recovery of muscle motor and sensory function will be described in some detail since the recovery of muscle power and proprioception are paramount to a good functional recovery.

CHAPTER 2

GENERAL MATERIALS AND METHODS.

The following chapter outlines those methods which are common to all experiments presented in this thesis. The methods specific to a particular experiment are given in detail in the appropriate chapter. Details of reagents used are given in appendices at the end of the thesis. The experimental model used was the sciatic nerve of the adult albino rat. The muscle selected for detailed assessment was the extensor digitorum longus (EDL) which is supplied by the peroneal branch of the sciatic nerve.

2.1 Anaesthetic Technique

All experiments were performed under general anaesthesia. The method used was the same in every case. The rats were anaesthetised with 0.5ml/kg of a one to one mix (by volume) of Hypnorm (fentanyl 0.315mg/ml and fluanisone 10mg/ml, Janssen Pharmaceuticals Ltd, Grove, Oxford.) and Hypnovel (midazolam hydrochloride 5mg/ml, Roche Products Ltd, Welwyn Garden City, Herts.) injected intramuscularly into the right hindquarter. Care was taken to avoid the area where the sciatic nerve runs. The depth of anaesthesia was considered to be adequate when the animal no longer responded to pinching of its footpad or to touching the cornea.

2.2 Methods of Nerve Repair

Three methods of nerve repair were examined.

1) Direct Nerve to Nerve (N-N) repair by epineurial suture.
2) Repair by coaxial, freeze-thawed, autologous muscle graft.
3) Repair by three-strand cable graft.

The contralateral, normal leg was used as a control.

Male Sprague-Dawley rats of initial weight 250g were used in the first two groups. They were all of approximately the same age, *i.e.* young adults. Unfortunately it proved impossible to harvest enough autologous nerve from a single rat to fashion a three-strand cable graft since this would have meant paralysing at least two of its four limbs a situation which was not acceptable to the Home Office Inspector. This problem was solved by using inbred Lewis rats of comparable weight, sex and age to the Sprague-Dawley animals. One rat was sacrificed to provide enough isogenous nerve to graft four other rats. Rejection was not a problem since these rats were bred to be genetically similar. Myelinated axon counts in the sciatic and peroneal nerves and all physiological variables under study, including the regenerative response were measured in normal Lewis rats and compared to those of normal Sprague-Dawley rats. There were no significant differences between the two, as compared by Student's t-test. (Appendix 1)

The left hindquarter was shaved and cleaned with a

solution of chlorhexidine 0.5% in 70% ethanol. Surgery was performed under sterile conditions by one operator (LMM).

1) Method of direct N-N repair.

An incision, three to four centimetres long, was made in the skin overlying the sciatic nerve in the thigh. This exposed the connective tissue border between gluteus maximus and biceps femoris muscles. This avascular line was incised using dissecting scissors. Biceps femoris muscle was reflected from its origin along the pelvis and pulled distally to reveal the sciatic nerve from sciatic notch to the division into peroneal and tibial nerves (Figure 4).

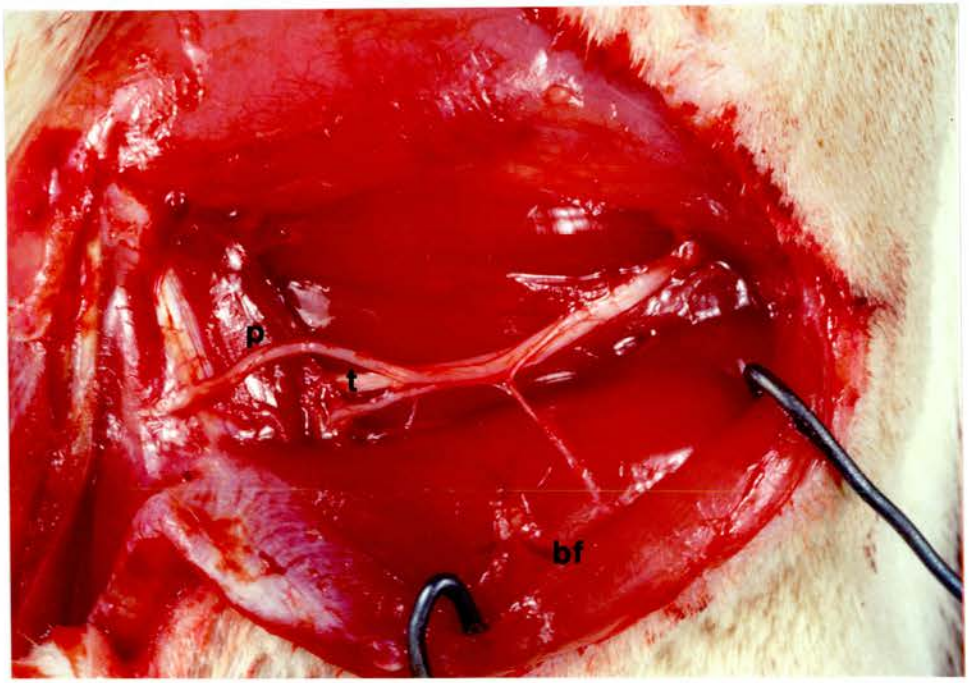


Figure 4: Dissection of the sciatic nerve of the rat x2.5
p=peroneal nerve, t=tibial nerve, bf=biceps femoris M.

The sciatic nerve was divided (using sharp, straight microscissors), 1 cm from the sciatic notch and a 0.5 cm section removed. Retraction of the ends left a gap of at least 1cm. Enough nerve was mobilised to ensure apposition of the cut ends without significant tension although excessive mobilisation was avoided in order to maintain the blood supply. The nerve was repaired using four to six 10/0 polyamide sutures (Ethilon, Ethicon Ltd, UK) through the epineural sheath. An operating microscope (Wild, Heerbruug.) and standard microsurgical technique was used. A piece of biceps femoris muscle was removed at this stage to ensure complete comparability with the muscle-grafted rats (see below). The remaining biceps femoris muscle was sutured to its origin using 6/0 Vicryl (Ethicon Ltd, UK.). The skin was closed with interrupted 6/0 Vicryl. The wound was again cleaned with chlorhexidine in alcohol and the rat left to recover.

2) Nerve repair using freeze-thawed muscle graft.

Biceps femoris muscle was reflected and the sciatic nerve divided as described above.

A strip of biceps femoris approximately 2cm by 0.75cm was removed, making sure that the muscle fibres were running parallel to the long axis. Enough fresh muscle had to be removed to allow for up to 50% shrinkage during freezing. Haemostasis was secured using diathermy. The piece of muscle was wrapped in aluminium foil for ease of handling and placed in a container of liquid nitrogen until thermal equilibrium was reached. The muscle was

then thawed quickly in distilled water at room temperature, still in its foil wrapping. This was removed when the muscle was completely thawed since it tended to stick to the muscle when it was frozen. The muscle was then trimmed to furnish a graft of appropriate length and diameter. The muscle fibres were always orientated coaxially with the axis of the nerve. The length of the graft was approximately 1cm in each case. The muscle graft was sutured between the proximal and distal stumps using 10/0 Ethilon epineural sutures and standard microsurgical technique (Figure 5). Subcutaneous tissue and skin were closed with 6/0 Vicryl as described above.

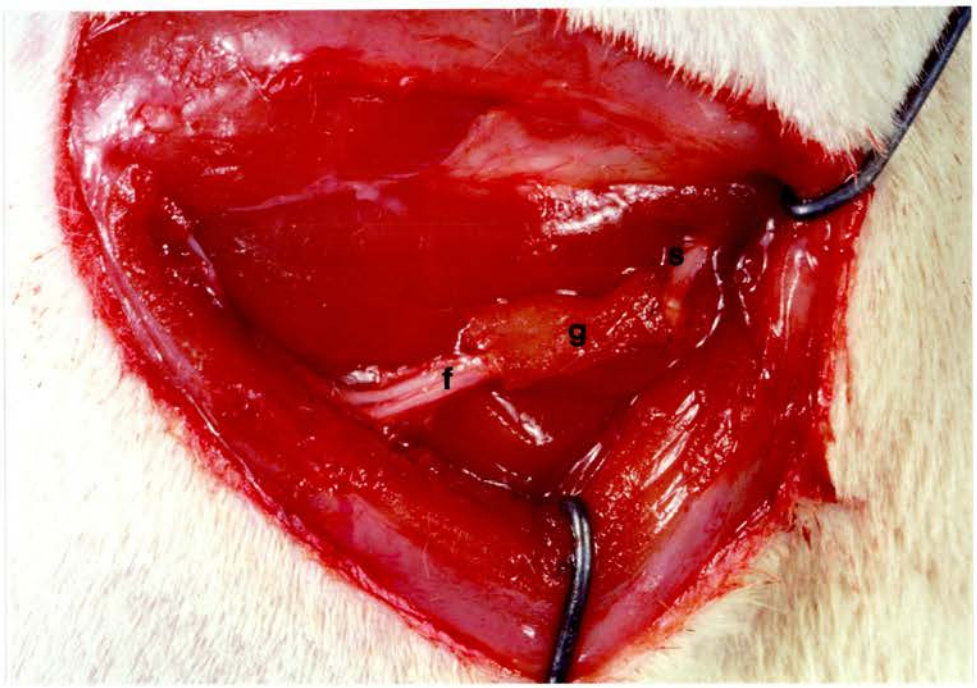


Figure 5: The muscle graft *in situ* x2.5
g = muscle graft, s = proximal sciatic N., f = tibial & peroneal fascicles.

3) Nerve repair using three-strand cable graft.

One Lewis rat was anaesthetised as described in section 2.2. Radial, ulnar and median nerves were harvested. It was possible to excise these nerves from their origin from the brachial plexus to mid-forelimb level. A length of approximately 5cm could be obtained. The rat was then killed by cervical dislocation. The nerves were cut into 1cm lengths using a sharp razor blade, being careful not to damage the fibres in any way. The grafts were stored for short periods, before use, in gauze swabs soaked in normal saline. Biceps femoris muscle was reflected and the sciatic nerve divided as described above.

Three lengths of harvested nerve (one each from median, ulnar and radial nerves) were placed into the gap in the sciatic nerve. Preliminary axon counts had shown that a three-strand cable graft would provide enough endoneurial tubes to allow complete regeneration of all axons contained in the sciatic nerve (Appendix 2). It proved impossible to use a purely sensory nerve *e.g.* the sural nerve since axon counts had shown that this would involve a cable graft containing nine strands of nerve (Sural nerve myelinated axon count = 900, sciatic nerve count = 8000, approximate values). Many rats would have had to be sacrificed in order to provide enough graft material, and the grafts would have been very difficult, if not impossible to perform.

The sciatic nerve is bifascicular at this level, the largest of the two fascicles containing fibres destined for the tibial division and the smaller of the two

containing peroneal fibres (Figure 6).

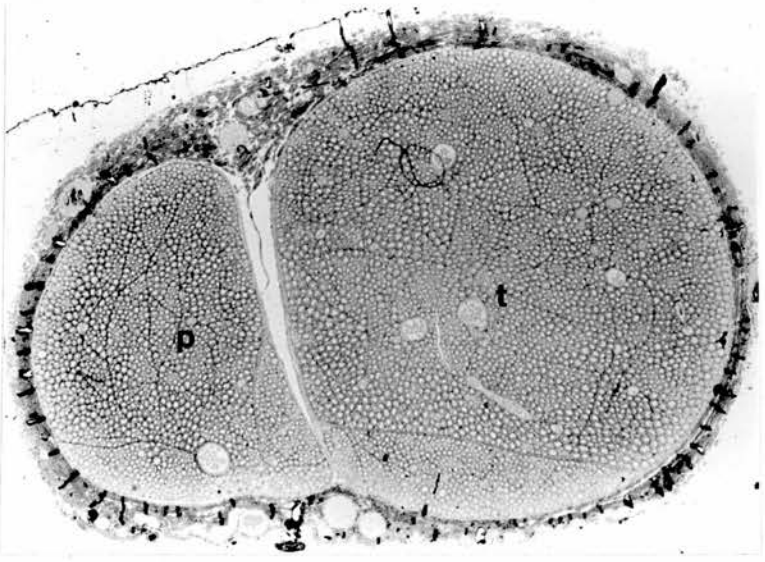


Figure 6: A semithin, plastic embedded section of the sciatic nerve showing the bifascicular structure x25. t = tibial and p = peroneal fascicles.

Two strands of cable graft were aligned with the tibial fascicle and one with the peroneal fascicle. The three strands were held together in a bundle with a drop of fibrin glue (Tisseel, Immuno Ltd, Artic House, Dunstane Green, Kent). (Further information is given in Appendix 3). One or two 10/0 Ethilon sutures were placed epineurally in each end to hold the graft in position but this was technically difficult because of the small diameter of the individual cable strands. These sutures also acted as markers for the graft. Fibrin glue was used to hold the bulk of the graft in place. Care was taken to ensure that no glue was placed on the cut

surfaces of the nerves. A piece of biceps femoris was excised for the reasons given above. The subcutaneous tissues and skin were closed as described previously.

In all cases, after operation the rats showed the expected signs of complete denervation in the left sciatic nerve territory, namely, disturbance of gait due to muscle denervation and complete anaesthesia of the limb. All qwounds healed well and there were no cases of wound infection. The perioperative mortality rate was 3.5% All of these deaths were related to anaesthesia and all occurred within 24 hours of surgery. Some rats developed sores and self-mutilated feet on the operated side owing to loss of sensation. These were treated with antibiotic spray (Tribiotic). All had healed by 50 days when sensation was beginning to return. The rats were housed in the Faculty animal house, three to four to a cage and were given free access to food and water. They were allowed to mobilise freely. The food used was an ageing rat and mouse diet (Bantin and Kingman) designed to prevent excessive weight gain.

2:3 Processing for Semithin Sections

See Appendix 4 for solutions.

1) Nerve specimens approximately 1cm in length were placed in 4% cacodylate buffered glutaraldehyde (pH 7.4) at 4°C for 1 hour. The ends were trimmed carefully with a razor blade and discarded. The remaining specimens were carefully cut into 1mm thick transverse slices. The initial fixation made the tissue firm enough to be

handled in this way. The 1mm slices were then fixed in fresh buffered glutaraldehyde for a further hour. They were subsequently washed in sucrose buffer wash solution for 1-1.5 hours. The tissue can safely be left overnight in sucrose buffer if need be.

2) The specimens were incubated in 1% cacodylate buffered osmium tetroxide (TAAB Ltd.) for 2 hours at room temperature. The time at this stage was not critical unless the blocks were to be used for ultrathin sections as well. In that case 2 hours is the recommended maximum. Less than 2 hours resulted in poorly fixed myelin and obvious artefact.

3) The specimens were washed in 10% ethanol for 60 minutes (2 x 30 minute changes), followed by 3 washes in absolute ethanol for 30 minutes each.

4) The specimens were placed in propylene oxide for 30 minutes and then in Araldite for at least 1 hour, although they can safely be left overnight at this stage at room temperature.

Stages 3) and 4) were carried out in a Lynx e1 microscopy tissue processor. (Australia Biomedical Corporation, Melbourne.)

5) The specimens were removed from the processor, placed in plastic moulds, infiltrated with fresh Araldite at room temperature and left overnight.

6) Finally the specimens were placed in fresh Araldite, having orientated them in the plastic moulds, and placed in an oven at 60°C for polymerisation of the plastic for at least 48 hours.

Semithin (1μm) sections were cut from the blocks using a glass knife and a Reichart-Jung OMU-3 microtome. The sections were dried flat on glass slides on a hotplate and stained with 1% Toluidine Blue in 1% sodium tetraborate (BDH Ltd). This was left on the section for approximately 15-20 seconds and then washed off in running tap water. The stained sections were air dried and stored unmounted in the dark.

2:4 Nerve Fibre Counts and Myelin Sheath Thickness

Morphometric analysis was performed using the Vids III System. Semithin (1μm), plastic embedded sections (prepared as described in section 2:3) were placed under the x100 objective oil immersion lens of a microscope (Leitz, Germany). The total magnification was x800. This image was digitised and viewed on a VDU screen. Measurements were made using a cursor and a digitising tablet. Data handling was carried out by an IBM XT computer interfaced to the Vids III system and to a peripheral plotter. The magnification had already been calibrated with a stage graticule. This system was capable of simple point counting, measuring areas and volumes, and measuring the distance between two points.

It was used to determine the absolute numbers of myelinated axons present in each section. No attempt was made to measure unmyelinated axons. The system of absolute counts was used, despite being lengthy, since it was felt to be the most accurate and the least susceptible to observer bias. If a sampling method is used it is assumed that the fibre density per unit area is uniform throughout a nerve, but in a large nerve like the sciatic, this is not the case. This assumption is also particularly suspect in the graft, where fibre density can be quite variable. The counting error using this method (the difference between repeated counts of the same specimen by the same observer) was 1.5%

Maturation was assessed by measuring myelin sheath thickness, using the two dot system on the Vids apparatus. The fibres measured were chosen at random by counting all fibres in each field which fell into a defined rectangular area, 10cm by 5cm, drawn onto the VDU screen. Because of the small distances involved, despite the highest magnification, this measurement was intrinsically relatively inaccurate. Large numbers of such measurements were made (at least 400 in each experimental group), enabling trends to be seen, even if the results had to be interpreted with great care.

2:5 Statistical Analysis

The statistics of all small samples were dealt with by hand using a scientific calculator with statistical functions (Texas Instruments). Larger sample statistics

were calculated using a Tandon PC (Tandon Corporation, Chatsworth,CA, USA) and Lotus "Symphony" plus @stats software (Lotus Development Corporation, Cambridge). Samples were compared using Student's t-test unless otherwise stated. P-values of less than or equal to 0.01 were considered to be significant. Graph error bars display the standard error of the mean (SEM). Other statistical abbreviations used throughout include: $\bar{x}$ = mean, SD = standard deviation.

Graphs were plotted using "SlideWrite Plus" graphics software (Advanced Graphics Software, Inc. California, USA).

CHAPTER 3

REGENERATION OF MUSCLE AND NERVE

3:1 Recovery of function after nerve injury and repair depends on a) the number of fibres reaching the periphery, b) the speed with which new fibres reach the periphery so that degeneration of end organs does not occur, c) the maturation of new fibres and d) the appropriateness of the connections made. Many authors have concentrated on anatomical indices of nerve regeneration as methods of determining how effective various methods of nerve repair have been. The variables measured have included:

1) Nerve fibre counts (Greenman, 1913; Gutmann and Sanders, 1943; Glasby, Gschmeissner, Hitchcock *et al*, 1986a,b,c).
2) Myelin sheath thickness (Sanders, 1948, Glasby, Gshmeissner, Hitchcock *et al*, 1986b,d).
3) Fibre diameter spectrum (Sanders and Young, 1944, 1947; Hammond and Hinsey, 1945; Glasby, Gilmour, Gschmeissner *et al*, 1990).
4) Internodal length (Hiscoe, 1947; Vizoso and Young, 1948; Hildebrand, Koscis, Berglund *et al*, 1985).

The evidence accumulated from these and other studies has been supported by electrophysiological methods, in an attempt to make a fuller assessment of recovery (Berry, Grundfest and Hinsey, 1944; Cragg and Thomas, 1964; Takano 1976; Gattuso, Glasby & Gschmeissner, 1988; Glasby, Gattuso and Huang, 1988). It should be borne in mind that, in the human, anatomical and

electrophysiological evidence of recovery may not correlate with the patient's subjective appraisal of the degree of recovery. Nevertheless, since most research work is done on laboratory animals, objective methods of assessment as described above have much to offer.

The changes associated with neurotomy and repair can be summarised as follows.

1) Increased Numbers of Small Fibres.

Initially, axon outgrowth was by the development and elongation of unmyelinated axon sprouts. Each axon in the proximal stump could contribute up to six to twenty sprouts which explained the large increase in fibre numbers seen after nerve repair (Aird and Naffziger, 1939; Aitken, Sharman and Young, 1947; Aitken and Thomas, 1962; Bray and Aguayo, 1974). Numbers eventually decreased with time as sprouts with no peripheral connections died back (Aitken, Sharman and Young, 1947). It is because of this that absolute fibre counts after nerve repair can be so misleading as vigorous sprouting, with large numbers of fibres in the distal stump, may mean nothing if appropriate end organ contacts are not made.

2) Decreased Fibre Diameter.

This paralleled the increase in fibre numbers. As fibres died back the remaining fibres increased in size as maturation took place. The eventual size reached by regenerating nerve fibres was dependent to some degree on the diameter of the parent fibre but mainly on whether contact with an appropriate end organ had been made

(Gutmann and Sanders, 1943; Sanders and Young, 1944; Hammond and Hinsey, 1945; Weiss, Edds and Cavanaugh, 1945; Aitken, Sharman and Young, 1947; Aitken and Thomas, 1962; Bray and Aguayo, 1974). Fibres which connected with an inappropriate end organ were able to be myelinated and to grow but never regained their full size (Sanders and Young, 1947). The size of the distal stump (or graft) endoneurial tubes was also thought to play a part in determining the eventual size of the nerve fibre. It has been suggested that endoneurial tubes which were shrunken or surrounded with collagen could impede circumferential fibre growth (Sanders and Young, 1944; Weiss, Edds and Cavanaugh, 1945). This is particularly relevant when cable grafts are used since the nerves used for the graft are usually sensory nerves and will have small diameter fibres. The resulting endoneurial tubes will be smaller than many large motor fibres (see table 3:1) which may result in the loss of these large diameter fibres. The muscle graft has an advantage here in that the BM tubes which it furnishes are of much larger diameter (see chapter one) and should not have a constricting effect on maturing nerve fibres.

3) Myelin Sheath Maturation.

Maturation of the myelin sheath increased with time after nerve repair but never returned to normal values even after 300 days in the rat (Gattuso, Glasby and Gschmeissner, 1988). Even if axons did not make contact with end organs they could still be myelinated (Aitken,

Sharman and Young, 1947). Myelin thickness was shown to increase with increasing fibre diameter (Friede, Brzoska and Hartmann, 1985).

Table 3:1 Classification of Muscle Efferent Fibres

Type of Sheath	Myelinated				Non-myelinated	
Fibre Diameter(μm)	22				1.5	2 - 0.1
Conduction Velocity (m/s)	100	60		30	4	0.5
Erlanger and Gasser Classification	Aα	Aß	Aγ		B	C
	skeletomotor				autonomic	
		fusimotor				

Erlanger and Gasser (1937)

In the normal nerve, internode length has been shown to vary with the diameter of the fibre (Hiscoe, 1947; Vizoso and Young, 1948). After regeneration this relationship changes. All sciatic nerve fibres in the rat had the same internodal length (approx 300μm) after a crush injury (Hildebrand, Kocsis, Berglund *et al*, 1985; Hildebrand, Mustafa, Bowe *et al*, 1987; Gattuso, Glasby and Gschmeissner, 1988).

These changes were more severe in nerves repaired by epineurial suture than in nerves which had undergone a simple crush injury (Gutmann and Sanders, 1943).

The effect of cable grafting on nerve function has yet to be looked at in the rat. Most authors in the past have simply looked at the effects of full thickness autologous nerve graft, usually by reversing a segment of nerve or taking a graft from the opposite side. This of course

almost never happens in the clinical situation. Jenq and Coggeshall, (1987) have looked at eight millimetre gaps in rat sciatic nerve repaired with two-strand autologous sural nerve graft enclosed in silicon tubes. Surprisingly axon counts were not significantly reduced in the distal stump, compared to normal, eight weeks after repair. This may reflect axon branching or may have been related to the use of a silicon tube. Glasby, Gilmour, Gshmeissner *et al*, (1990) compared nerve fibre counts and fibre diameters in the femoral nerve of sheep after repair with muscle graft and cable graft and found no significant difference between them but did not go on to examine physiological recovery. In the following study rat sciatic nerve repaired with freeze-thawed autologous muscle graft will be compared to nerves repaired with cable graft and direct epineurial suture, using anatomical and physiological indices of recovery. Since it could be argued that recovery in the nerve itself is of no consequence without appropriate end-organ connections and end-organ function, it seemed logical to extend the study to an assessment of muscle recovery in the same rats.

3:2 Materials and Methods

Three methods of nerve repair were examined.

1) Direct N-N repair by epineurial suture.
2) Repair by coaxial, freeze-thawed, autologous muscle graft.
3) Repair by three-strand cable graft.

The contralateral, normal leg was used as a control. The muscles of the contralateral leg after nerve injury might undergo hypertrophy consequent upon an increase in workload. This was tested by comparing muscle mass and maximum isometric twitch in four normal, unoperated, age-matched controls, with the normal side in groups of rats which had undergone nerve repair by various methods, 150 days previously. There were no significant differences between the groups (Appendix 5). It was felt that the contralateral leg was therefore a legitimate control.

There were 16 rats in each group. Rats were reviewed at 50, 100, 150, and 300 days after operation.

3:2:1 Electrophysiological Recording (Figure 7)

At review the rat was weighed and anaesthetised as previously described. The repaired sciatic nerve was exposed as described for nerve repair. The nerve was dissected free of connective tissue and fat and exposed for recording. It was much more difficult to dissect the nerve free of connective tissue on the operated side. It was noted that there seemed to be more connective tissue surrounding the cable grafted nerves than around the nerves repaired by the other methods. In most cases an intravenous cannula was introduced into the right jugular vein for infusion of fluids and anaesthetic as required. A digital temperature probe was inserted into the rat's rectum to measure its core temperature. The rat's core temperature was maintained as close to 37°C as possible

by having it lie on an electric blanket. The nerve and surrounding tissues were kept moist with normal saline at 37°C.

A bipolar, low impedance silver wire stimulating electrode was placed under the sciatic nerve proximal to the repair, just as the nerve left the sciatic notch, being careful not to stretch the nerve or to let the electrode touch the underlying tissues. The cathode was placed distally. A similar recording electrode was placed under the peroneal division of the sciatic nerve just before it passed to the lateral side of the knee joint. A silver wire ground electrode was placed in the muscle underlying the sciatic nerve. Supramaximal stimuli of 0.2 - 0.3msecs duration and 2 Volts amplitude at 0.5Hz were applied to the nerve using a Dagan 9200 Omni Pulse stimulator (Clark Electromedical Instruments, Reading.) The resulting compound action potential was displayed on a 4070 Digital Storage Oscilloscope (Gould Electronics), with a delay of 10 msec triggered by a TTL output from the stimulator, after passing through a Neurolog NL104A AC amplifier and NL125 filter (Digitimer Ltd, Welwyn Garden City, AL7 1AF). The averaged action potential was displayed (8 sweeps averaged by a Gould waveform processor, type 270). A square wave TTL timing pulse corresponding to the stimulus was also displayed. The time from the upstroke of the timing pulse to the various positive peaks of the averaged compound action potential was measured using cursors on the oscilloscope screen. The distance between the midpoints of the

stimulating and recording electrodes was measured as accurately as possible and the conduction velocities of the main fibre groups calculated using the formula:

$S = D/T$ where S = conduction velocity

D = distance between electrodes

T = time from timing pulse to peak of the action potential.

The excitability of the nerve was estimated by obtaining a strength-duration curve. The stimulus duration was held constant and the stimulus intensity increased from 0-10 Volts until a compound action potential with two peaks was obtained. This voltage was recorded. The second velocity peak of the action potential was used becauses the first peak was inconsistent in timing and size and was easily confused with the stimulus artefact. This was repeated for 32 different stimulus durations, from 0.01 msec to 100 msec. The results were collected on disc using a Tandon A.T.computer, model 7002, (Tandon Corporation, Chatsworth, CA, USA.) using Lotus "Symphony" software. A standardised strength-duration curve was obtained by plotting a graph of log(stimulus duration) v volts/rheobasic voltage (see discussion). A value for chronaxie was obtained from the graph at a later date by plotting the graph using SlideWrite software. An exponential curve was fitted to the points by the computer and the equation of the curve was displayed. It was of the form

$$y = ae^{-bx}$$

where a and b are constants

Since chronaxie is the duration of stimulus required to excite a nerve at twice the rheobasic voltage (which was always equal to one on the standardised curve), log10 chronaxie could be calculated by solving the equation of the curve when y=2. The antilog of this value gave the chronaxie value in milliseconds.

The Extensor Digitorum Longus muscle (EDL) (Figure 8) was carefully exposed. Its distal tendons were cut and tied to the post of an isometric tension transducer (Harvard Apparatus #52-9503), using inextensible wire. The tension transducer had previously been calibrated using weights suspended from a small hook (see Appendix 5 for details). The muscle belly was freed as far as possible taking care to leave the muscle blood and nerve supply (from the peroneal) intact. The temperature of the muscle belly at the end of the experiment was measured with a temperature probe inserted into the muscle belly. The temperature was always in the range 32°-34°C. Large variations in temperature are known to cause variations in the rate of muscular contraction and for that reason were avoided (Gordon and Phillips, 1953). The leg was immobilised using steel pins which were forced through the knee and ankle joints into a cork board underneath the animal. The muscle was pulled to a constant length in each experiment (resting length plus one third of resting length) and kept at this length, since the force of contraction of a skeletal muscle varies as the initial length (Buller, Eccles and Eccles, 1960a).

A supramaximal stimulus was applied to the sciatic nerve as before at a stimulus rate of 0.5 Hz. and the average of eight isometric tension traces displayed on the oscilloscope. The duration of twitch, the peak twitch, the time to peak, time to 50% peak and area under the curve were measured (in relation to the timing pulse) using a Gould Waveform Processor (type 270) attached to the oscilloscope. These variables were measured separately before insertion of the EMG electrode because it caused slight drag on the muscle and interfered with muscle contraction.

A bipolar concentric needle electrode (Clark Electromedical Instruments, SNE-100) was inserted into the EDL muscle belly to record EMG activity during supramaximal nerve stimulation as described above. A ground electrode was placed on inactive muscle nearby. The signal was initially amplified by a Neurolog NL850 isolated preamlifier, then fed through an AC differential amplifier and filters as described previously. The EMG signal was integrated by a Neurolog NL705 RMS Integrator (Digitimer Ltd), designed to provide a rectified analogue signal representing the power of the input signal. The time constant of integration was kept constant at 20msec. EMG, integrated EMG, isometric tension and a timing pulse were displayed synchronously on four channels of the oscilloscope, triggered by the stimulator. Eight sweeps were averaged and the average displayed. The distance between the midpoint of the stimulating electrode and the tip of the EMG electrode was measured. The waveform

processor was used to calculate :

a) Time to first peak of the EMG trace (known as latency). This time represents the time taken for nerve conduction, neuromuscular transmission and conduction of the action potential through the muscle membranes to the recording electrode. Latency values become shorter with increasing time after injury (Hodes, Larrabee and German, 1948). Latency is a useful clinical measurement since it is easily performed and is easily standardised for each patient. (The stimulating and recording electrodes are placed in the same spot each time and this constant value D is divided by the latency to produce a velocity known as the motor latency, which can be compared from visit to visit.) Unfortunately D in this experiment was not constant due to differences in the size of the rats whose weights varied from 550g to 1.2kg. The stimulating electrode was placed in the same anatomical position in each rat, just distal to the sciatic notch. This ensured that the electrode was always proximal to the graft. The EMG electrode was placed in the middle of the muscle belly. The distance between the two was measured and used as the D value. The latency was obtained from the oscilloscope and a motor latency obtained as described above. This velocity was then used with a standard D of 50mm, using the formula in a different way, to obtain a standardised latency for D = 50mm. It was decided to use this method of standardisation between rats, rather than use a standard distance between the stimulating and recording electrodes in the experimental set-up, because

in the larger rats the stimulating electrode would have been distal to the graft site. It is known that conduction velocities vary between the proximal and distal segments of the same nerve, being slower in the distal segment (Caruso, 1985,1987), so one should maintain the proportions of each part of the nerve the same in each case. Obviously in big rats D is much longer with a corresponding increase in latency. This method of standardisation overcomes the problem.

b) Area under the EMG trace.

c) Area under the integrated EMG trace.

The averaged displays were printed out on paper by the oscilloscope as a permanent record (Figures 9-16).

The stimulating electrode was then removed from the sciatic nerve and placed directly and proximally onto the EDL muscle belly. The muscle nerve was disrupted. The muscle was stimulated directly (1msec, 5Volts) at a frequency of 0.5Hz. EMG activity was recorded as already described. The time to first peak of the muscle action potential was measured relative to the timing pulse and used to calculate the conduction velocity of the muscle action potential across the muscle membrane, using the formula :

$$S = D/T,$$

where S = conduction velocity of the muscle action potential, D = distance between stimulating and recording electrodes, and T = time to first peak of the action potential.

The normal, control side was evaluated in the same way.

3:2:2 Specimens for Morphology and Counts

Specimens of proximal sciatic nerve, graft, peroneal nerve, normal proximal sciatic nerve and normal peroneal nerve were taken for histology. They were processed for semithin sections as described in chapter two. Total myelinated fibre counts, myelin sheath thickness and myelinated fibre diameters were obtained using the methods outlined in chapter two.

The EDL muscles were removed and weighed to quantify the amount of muscle wasting when compared to controls.

3:3 Results

Figures 9-12 show the averaged compound action potential as it was recorded in normal, N-N suture, muscle or cable grafted nerves at 300 days after repair. Note that after repair the shape of the compound action potential was still abnormal even at 300 days. After cable or muscle grafting the amplitude of the action potential was also reduced. The lower trace shows a timing pulse corresponding to the stimulus.

Figures 13-16 show the averaged display of EMG, integrated EMG and isometric tension as it was recorded, in response to a supramaximal stimulus applied to the sciatic nerve, in the same rats as Figures 9-12. The shape of the EMG trace was still quite abnormal in the grafted animals even at 300 days after repair.

Figure 17 shows the number of myelinated fibres found in the peroneal nerve (distal to the graft or anastamosis) 300days after nerve repair by N-N suture, muscle or cable

graft. The p-values of the various comparisons are shown in table 3:2.

Figures 18 and 19 show the fibre diameters and the myelin sheath thickness of normal and regenerated peroneal nerves (same animals as in Figure 17). Tables 3:3 and 3:4 show the respective p-values.

Figure 20 shows the number of myelinated fibres found within the graft itself, 300 days after repair with either cable or muscle graft. This was compared to the number of myelinated fibres in a normal proximal sciatic nerve. The number of fibres found in both kinds of graft was significantly different from normal ($p<0.0005$) but they were not significantly different from each other. Table 3:5 gives the statistics of the fibre diameter spectrum found in each kind of graft and Table 3:6 gives the same data for the myelin sheath thickness within the grafts (all data at 300 days after repair). There was no significant difference between the mean fibre diameter found in each type of graft or in the degree of myelination within the grafts at 300 days ($p>0.15$). The fibre diameters were not significantly different between the distal stump and the grafts. However, the degree of myelination was significantly different in the muscle graft when compared to the peroneal nerve ($p<0.01$). There was no significant difference in the level of myelination found in the cable graft when compared to its distal nerve (peroneal).

Figure 21-24 show ogival curves (cumulated percentage frequency curves) for all conduction velocity peaks in

normal and repaired sciatic nerve. Curve A in these figures shows all data points while curve B shows an expanded area of curve A for better comparison. The frequency distribution of velocities is quite skewed and so it is statistically quite incorrect to try to compare these ogival plots with any statistical test as they stand but they are useful as visual indicators of differences.

Figure 25 shows the fastest conduction velocity peak found in normal sciatic nerve and at various times after repair. There was no statistical difference between any of the repaired nerves at any time after repair but all repaired nerves were statistically different from normal ($p<0.0005$).

Figure 26 shows the percentage of compound action potential fibre peaks conducting at greater than 30m/sec. Reference to Tables 3:1 and 4:1 shows that these are large diameter motor fibres innervating skeletal muscle and large sensory fibres from muscle spindles and tendon organs. Approximately 20% of fibre peaks in the normal sciatic nerve action potential conduct at this velocity. (Note: this is <u>not</u> the same as the percentage of fibres conducting at this velocity. Each fibre peak represents a group of fibres. The bigger the peak the more fibres contribute to it. The fibre peak velocity is an average velocity for that group of fibres.) Interestingly, nerves repaired by cable graft never regained a fibre peak velocity of greater than 30m/sec. The other groups showed fibre peak velocities greater than 30m/sec

although this was very variable among rats, as shown by the error bars. Because of this, any statistics would be unreliable and so none has been attempted. A larger population of rats needs to be studied to clarify the situation.

Figures 27-30 show mean standardised strength-duration curves for normal sciatic nerve and nerve repaired by N-N suture, muscle or cable graft at 50, 100, 150 and 300 days after repair. Figure 31 shows figures 27-30 simplified and plotted on the same axes, without error bars, for comparison. Figure 32 shows the mean value of chronaxie for each type of nerve repair at various times after repair. At 100 days after repair and longer, there was no significant difference between experimental groups or between experimental groups and normal nerve. At 50 days muscle grafted nerve was not significantly different from normal but N-N suture ($p<0.005$) and cable grafted nerves ($p<0.01$) were significantly different. The strength-duration curve and chronaxie values for N-N sutured nerves at 50 days was slightly biased by the values obtained from one rat. These were unusually high and possibly reflected nerve damage occurring at the time of dissection. This may also have been due to poor reinnervation secondary perhaps to vascular problems or faulty suturing. However, even although large voltages had to be used, muscle contractions could still be elicited showing that peripheral reinnervation had taken place, making this latter explanation unlikely.

Figure 33 and 34 show motor latency and standardised

latency values after various methods of nerve repair over 300 days after repair. Tables 3:7 and 3:8 show their respective p-values.

Figures 35-37 show the recovery, with time, of the duration of twitch, time to peak tension and time to 50% peak tension in EDL muscle after nerve repair. Tables 3:9 - 3:11 show their respective p-values.

Figures 38-41 show various measures of the force of contraction of EDL muscle after nerve repair. Figure 38 shows the maximum isometric tension achieved, figure 39 shows the area under the isometric myogram (known as time-tension integral), figure 40 shows the average isometric tension produced (known as the time tension index) and figure 41 shows the area under the integrated EMG. Tables 3:12 - 3:15 give the p-values for these.

Figure 42 shows the "velocity of conduction" across EDL muscle. This is in fact a measure of the time taken for the muscle action potential to travel across the muscle cell membrane, T-tubules and sarcoplasmic reticulum to a recording electrode. Table 3:16 gives the p-values.

Table 3:17 gives the statistics for EDL muscle weights while figure 43 displays EDL muscle weights as a percentage of normal. The p-values are given in table 3:18

Figures 44-51 show histological details of muscle and cable grafts and normal nerves.

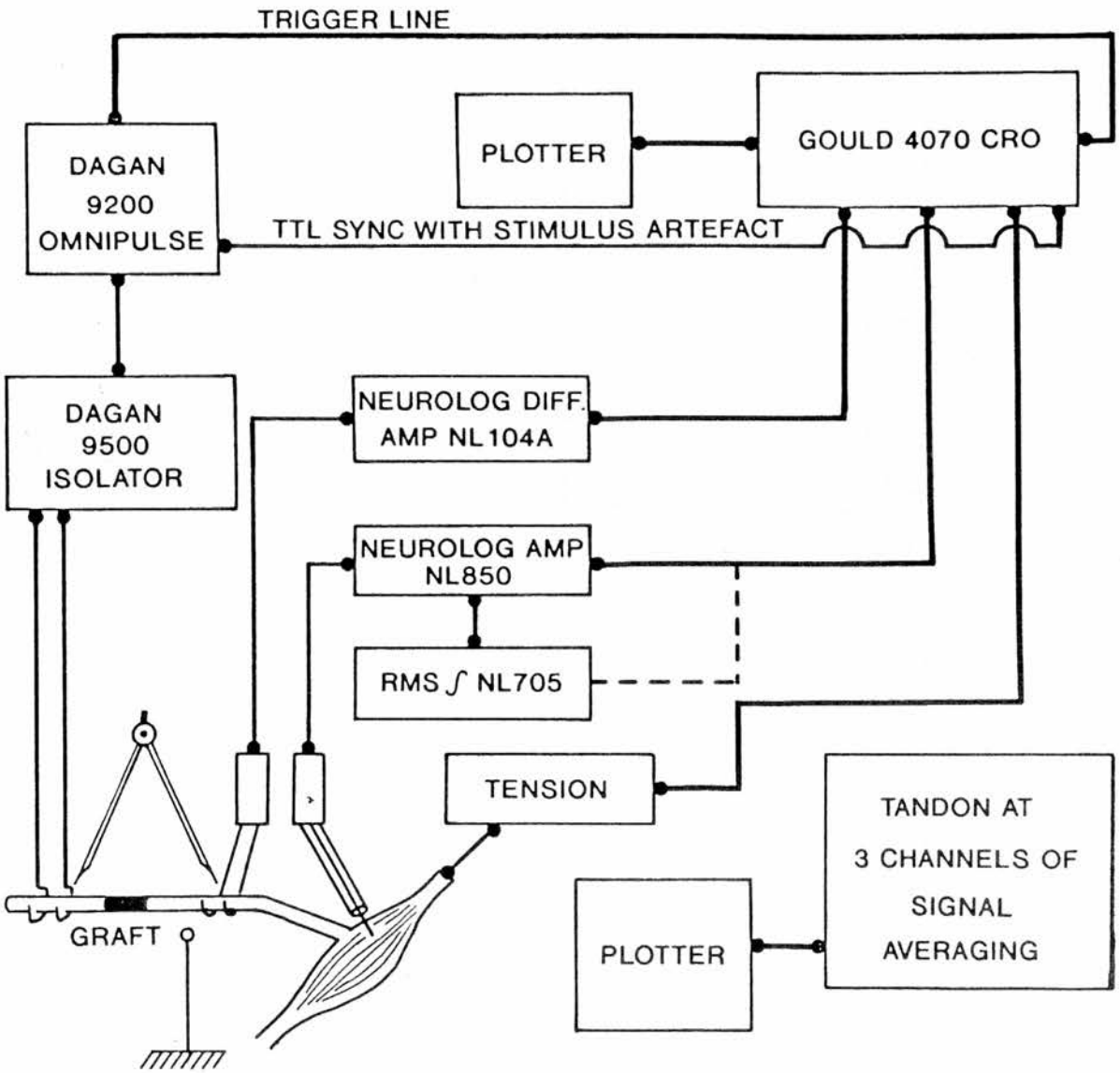


Figure 7: The electrophysiological recording set-up.

See text for details.

Figure 8: Dissection of EDL muscle in the rat. x 2.5

E = EDL muscle, T = tibialis anterior muscle,

P = the peroneal nerve at the knee. Note the multiple small nerve branches to the muscle.

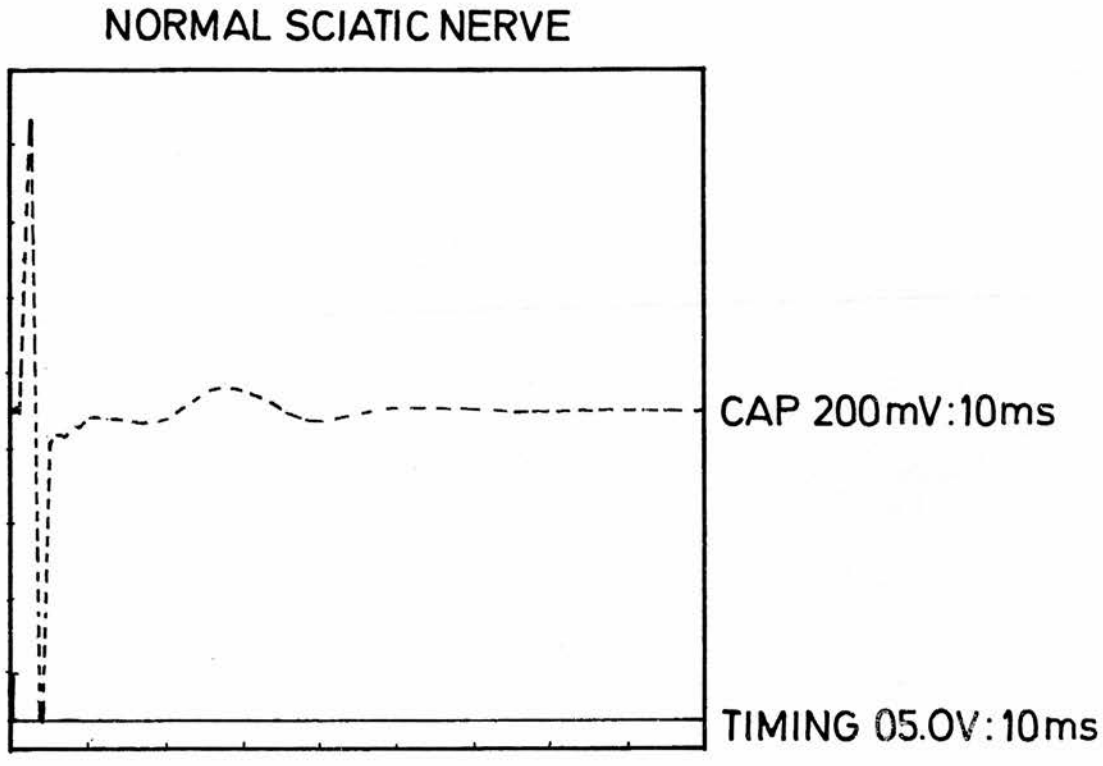


Figure 9: An averaged compound action potential, recorded extracellularly from normal sciatic nerve. (8 sweeps)

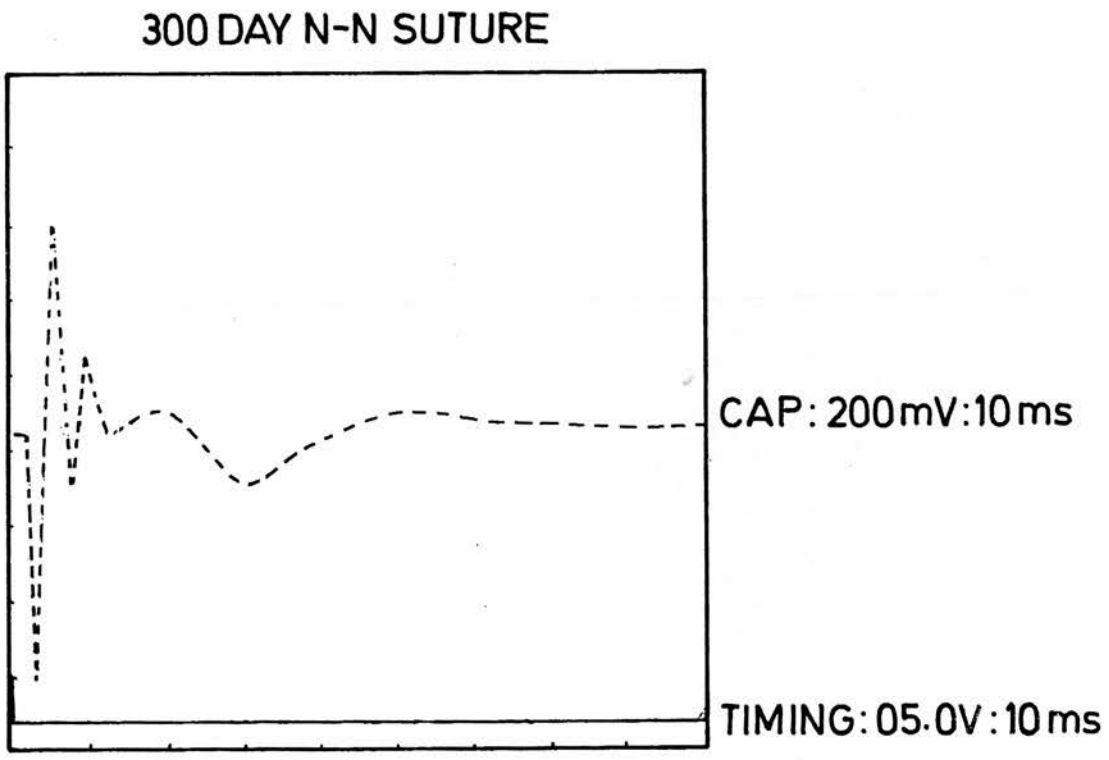


Figure 10: An averaged compound action potential, recorded extracellularly from sciatic nerve repaired by direct nerve suture. (300 days, 8 sweeps)

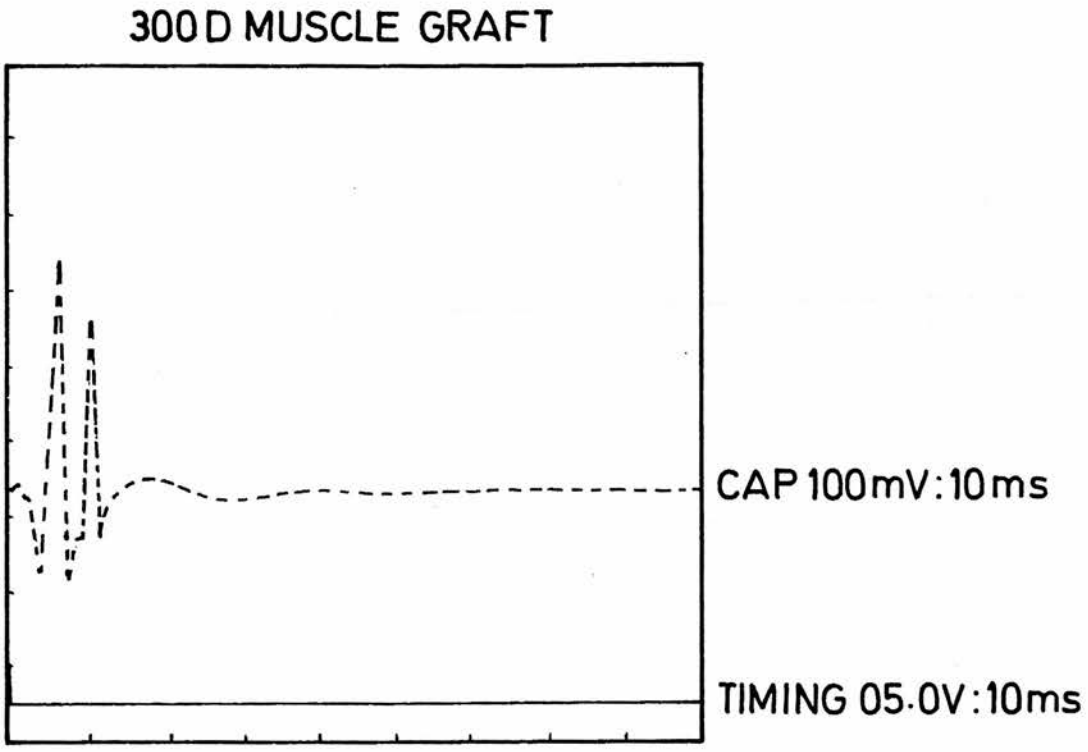


Figure 11: An averaged compound action potential, recorded extracellularly from sciatic nerve repaired with muscle graft. (300 days, 8 sweeps)

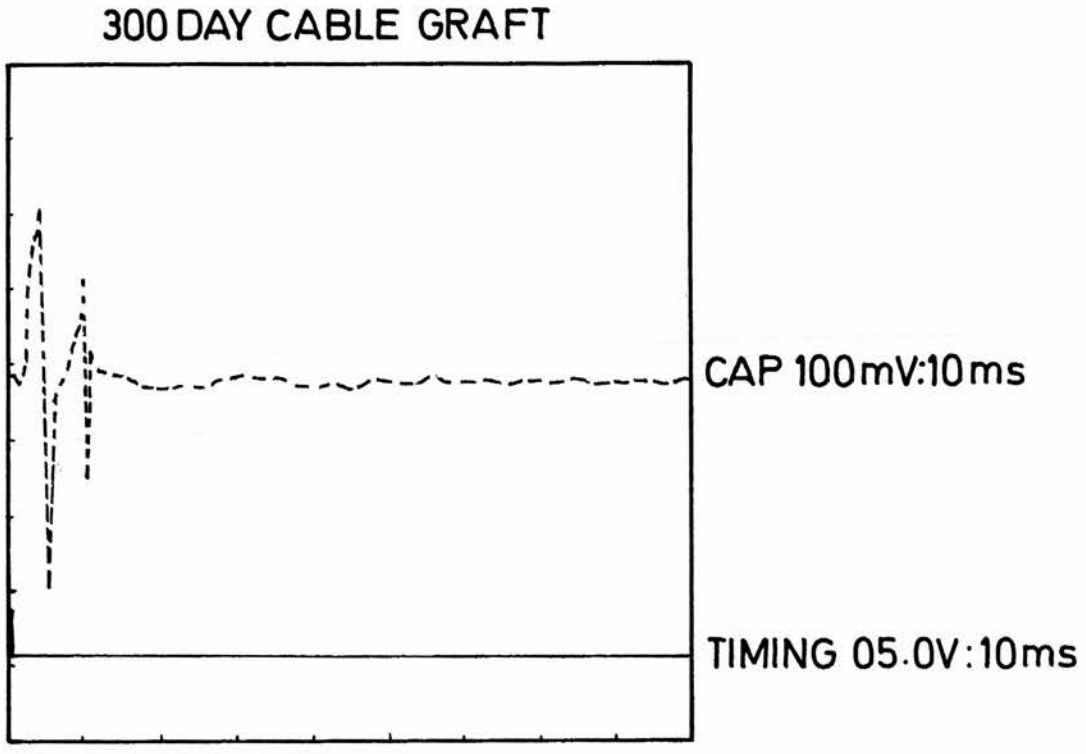


Figure 12: An averaged compound action potential, recorded extracellularly from sciatic nerve repaired with three-strand cable graft. (300 days, 8 sweeps)

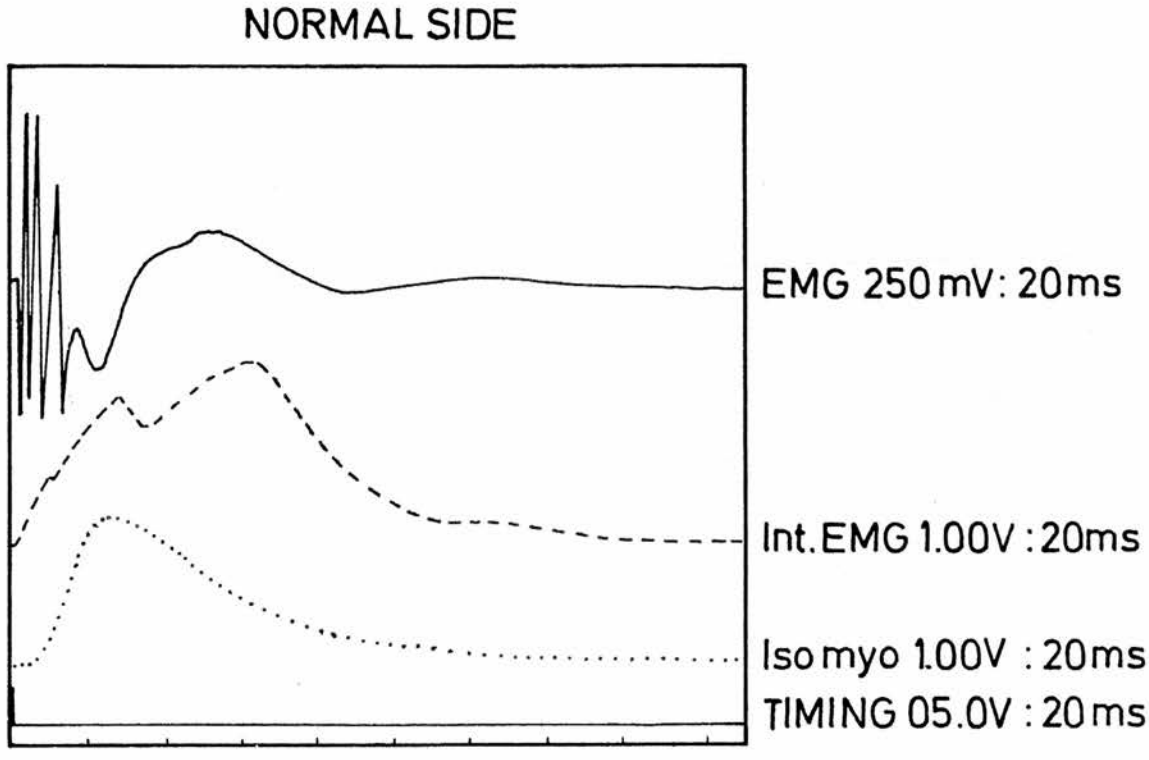


Figure 13: Averaged display of EMG, integrated EMG and isometric tension in normal EDL muscle in response to a supramaximal stimulus of the sciatic nerve. (8 sweeps)

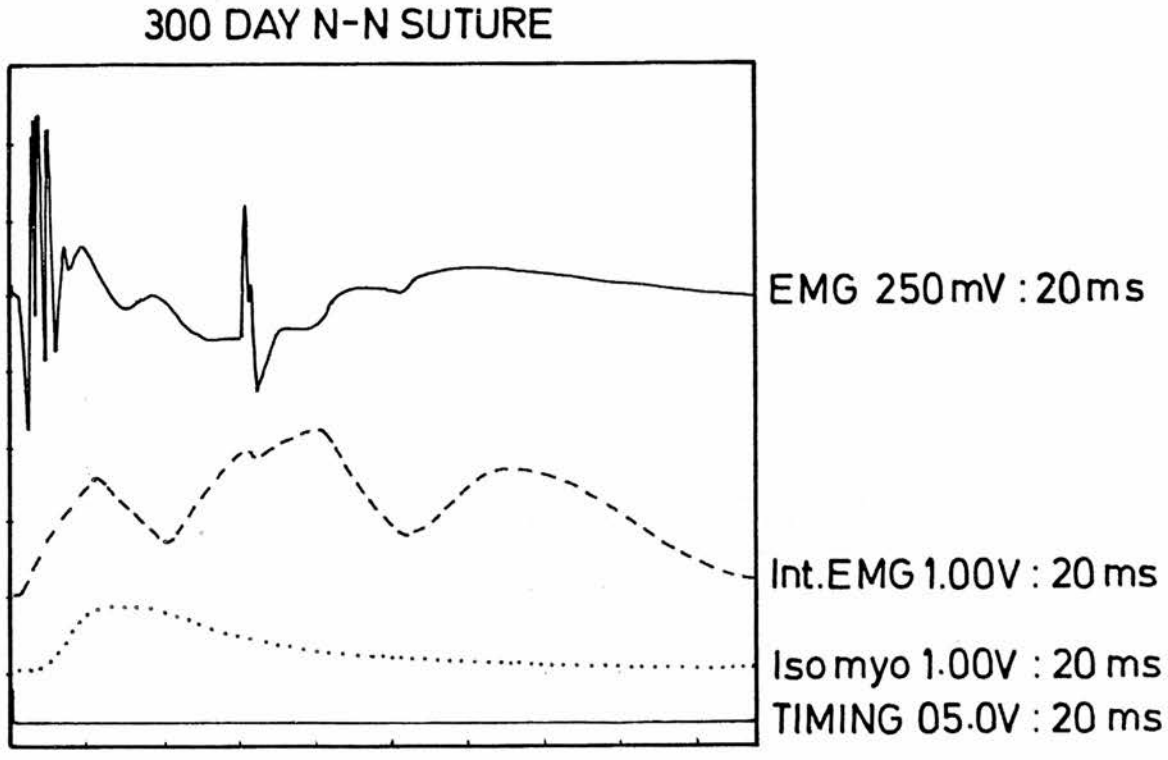


Figure 14: Averaged display of EMG, integrated EMG and isometric tension in EDL muscle, 300 days after repair of the sciatic nerve by N-N suture. (8 sweeps)

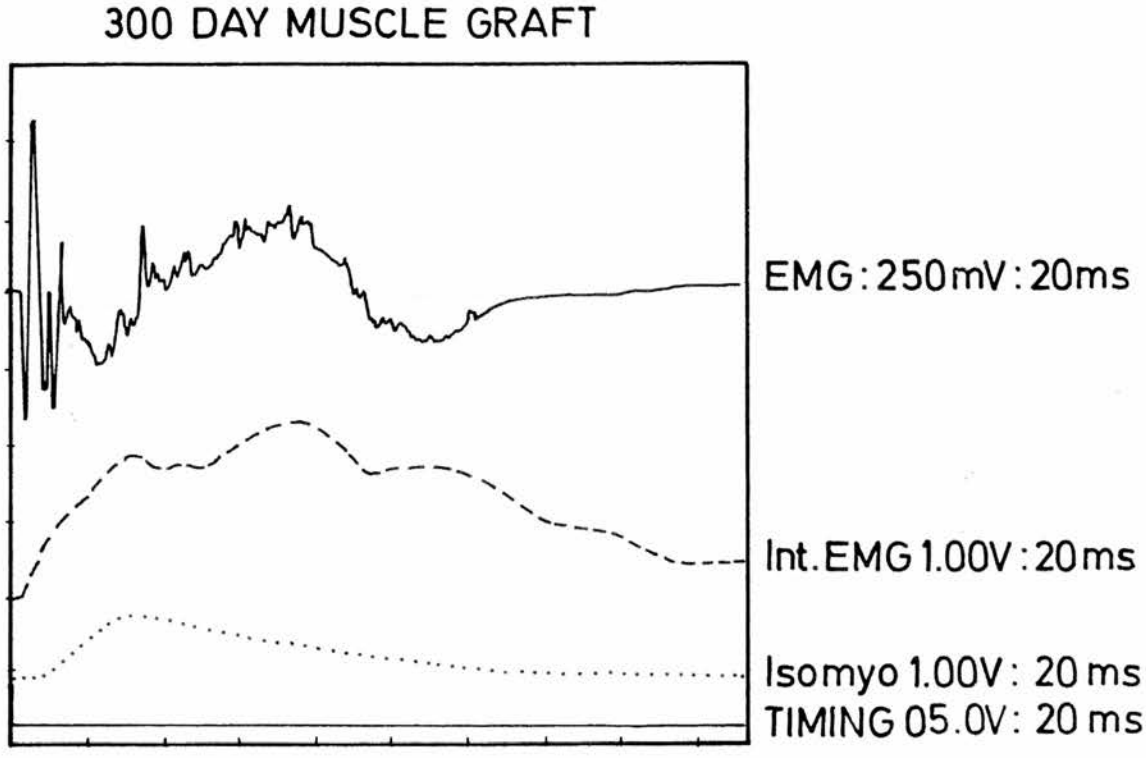


Figure 15: Averaged display of EMG, integrated EMG and isometric tension in EDL muscle, 300 days after repair of the sciatic nerve with muscle graft. (8 sweeps)

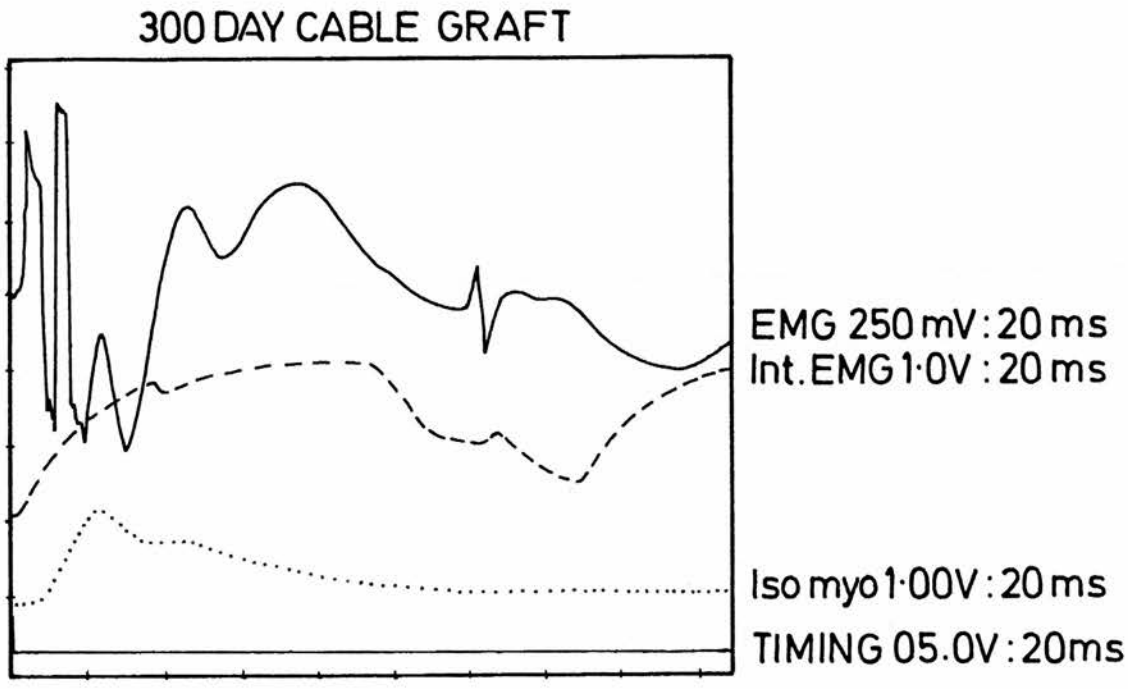


Figure 16: Averaged display of EMG, integrated EMG and isometric tension in EDL muscle, 300 days after repair of the sciatic nerve with cable graft. (8 sweeps)

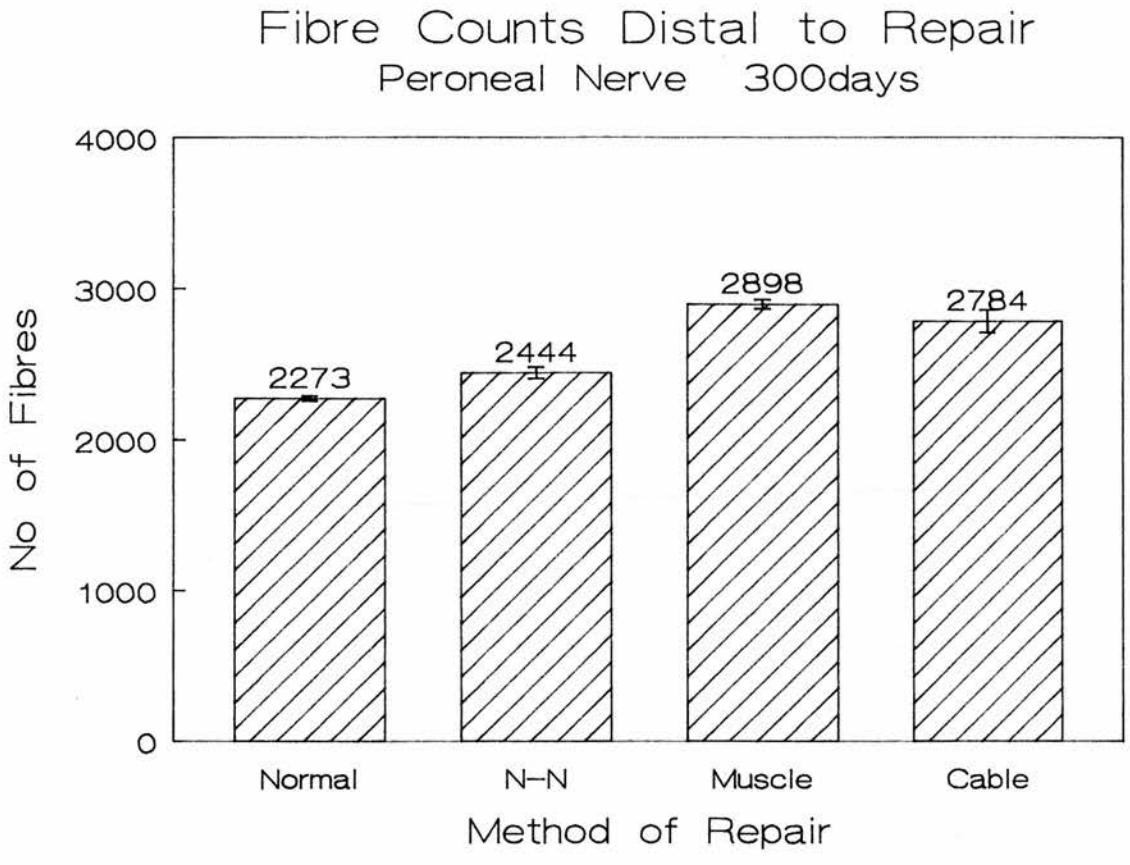


Figure 17: Myelinated fibre counts distal to the site of repair (peroneal nerve, 300 days after repair of the sciatic nerve).

Table 3:2 P – Values for Figure 17

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.0005	p<0.0005
N–N	NS	*	p<0.0005	p < 0.01
Muscle	p<0.0005	p<0.0005	*	NS
Cable	p<0.0005	p < 0.01	NS	*

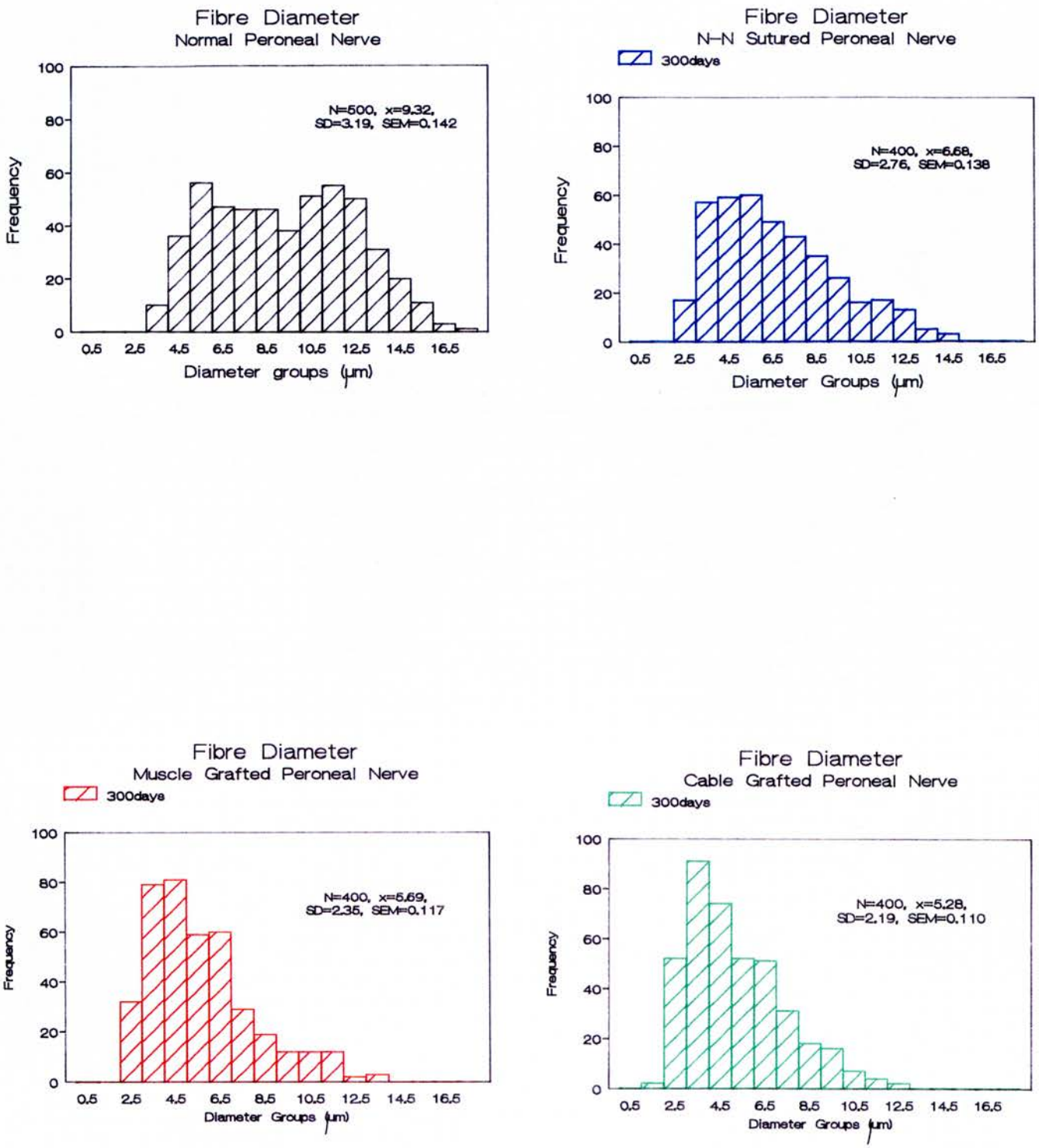


Figure 18: Fibre diameters in the peroneal nerve, 300 days after sciatic nerve repair by various means, compared to normal.

Table 3:3 Fibre Diameter P–Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	p<0.0005	p<0.0005
Muscle	p<0.0005	p<0.0005	*	NS
Cable	p<0.0005	p<0.0005	NS	*

Table 3:4 Myelin Sheath Thickness Distal to the Graft
P – Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	p<0.0005	p<0.0005
Muscle	p<0.0005	p<0.0005	*	NS
Cable	p<0.0005	p<0.0005	NS	*

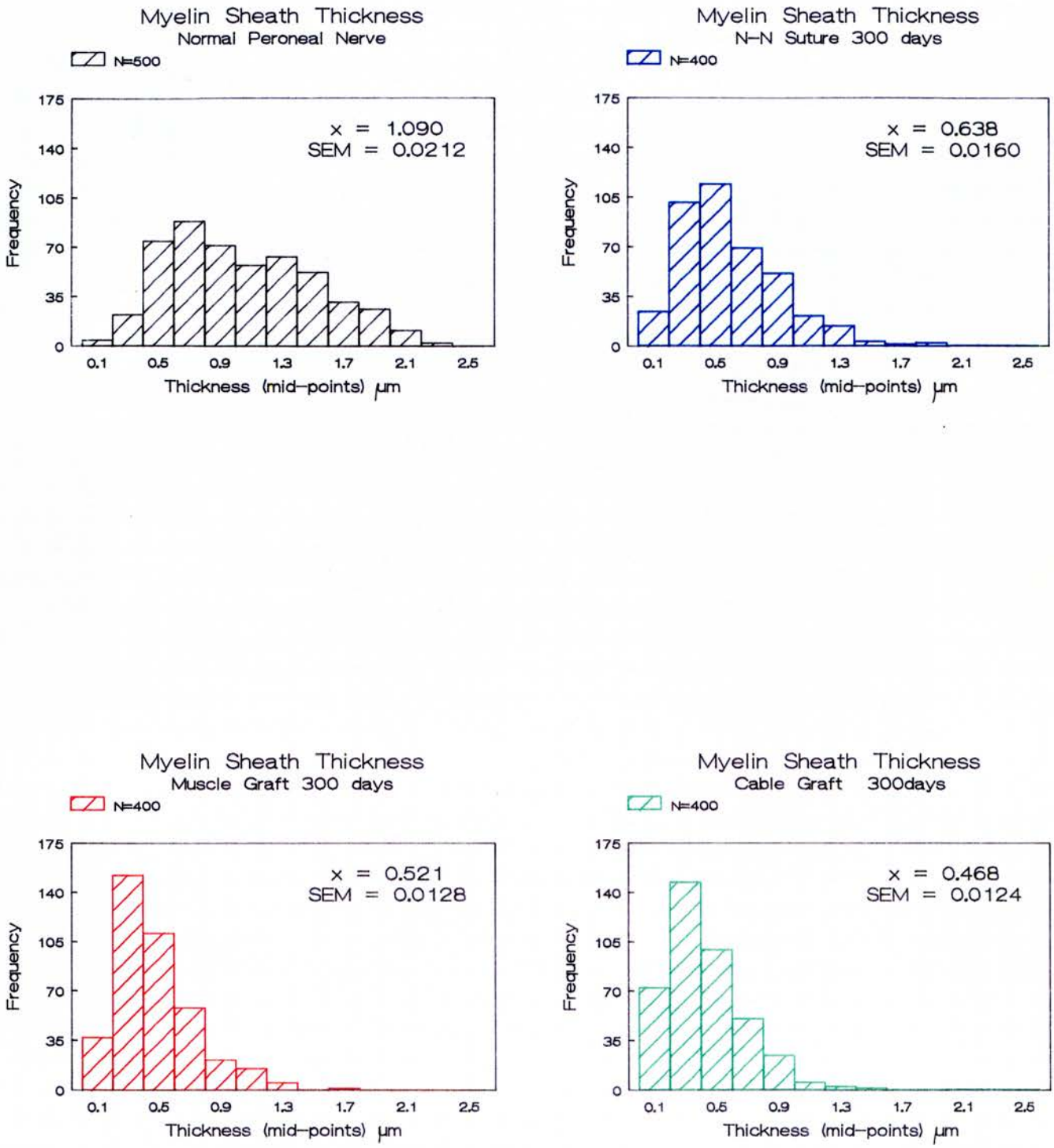


Figure 19: Myelin sheath thickness in the fibres of the peroneal nerve, 300 days after repair of the sciatic nerve by various means, compared to normal.

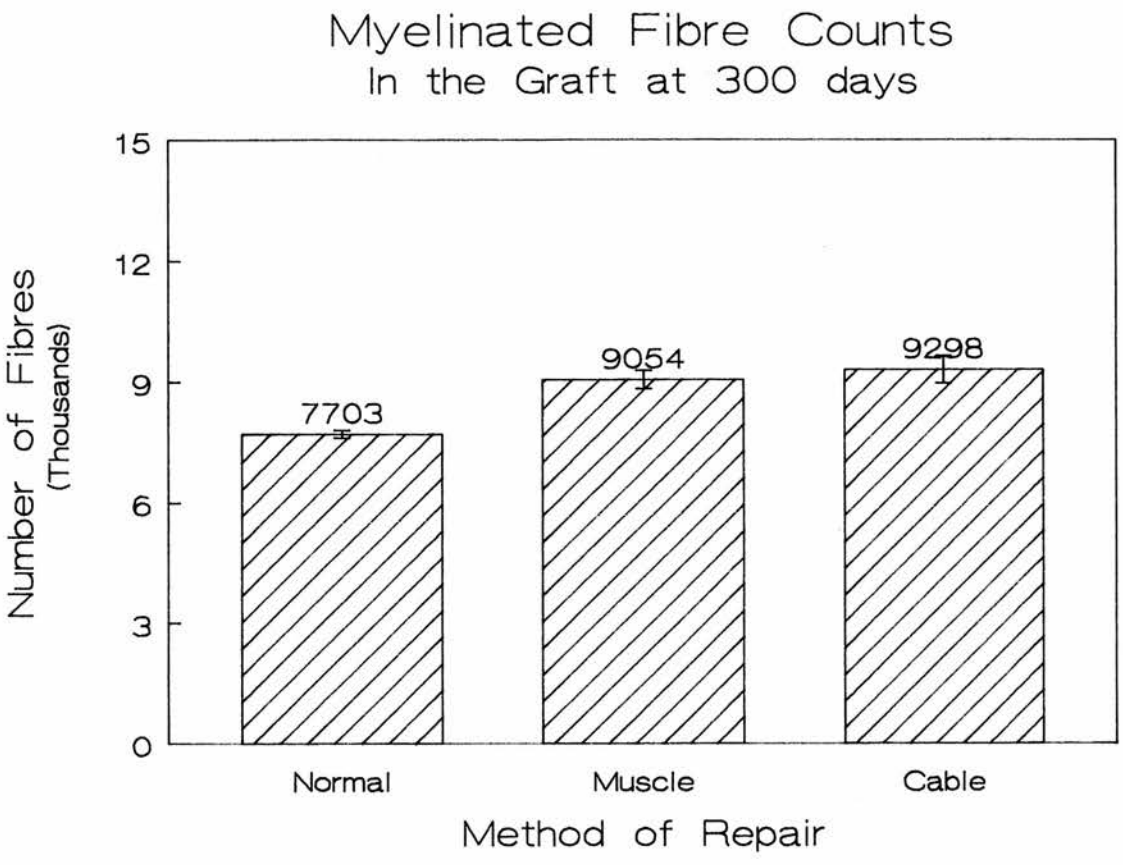


Figure 20: Myelinated fibre counts in muscle and cable grafts at 300 days, compared to normal proximal sciatic nerve.

Table 3:5 Fibre Diameters in the Graft (μm)

Muscle Graft	Cable Graft
N=400	N=400
x=6.01	x=5.74
SD=2.79	SD=3.01
SEM=0.140	SEM=0.151

Table 3:6 Myelin Sheath Thickness in the Graft (μm)

Muscle Graft	Cable graft
N=400	N=400
x=0.459	x=0.498
SD=0.247	SD=0.254
SEM=0.0124	SEM=0.0127

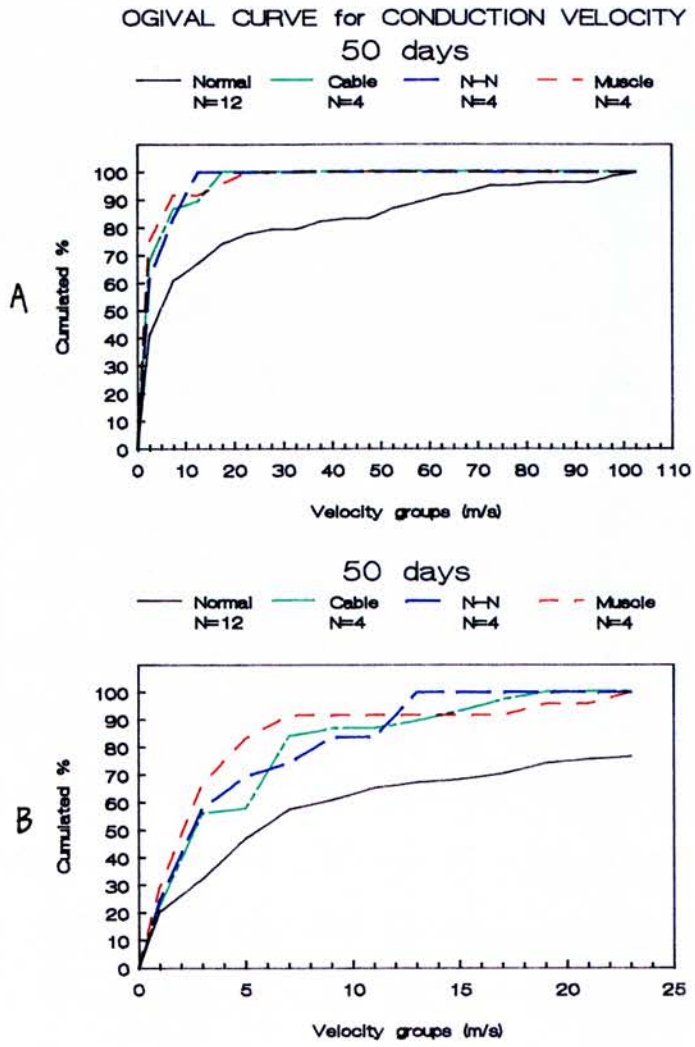


Figure 21: Ogival curves for conduction velocity in normal and repaired sciatic nerves at 50 days.
A: All values. B: Values up to 25 m/sec.

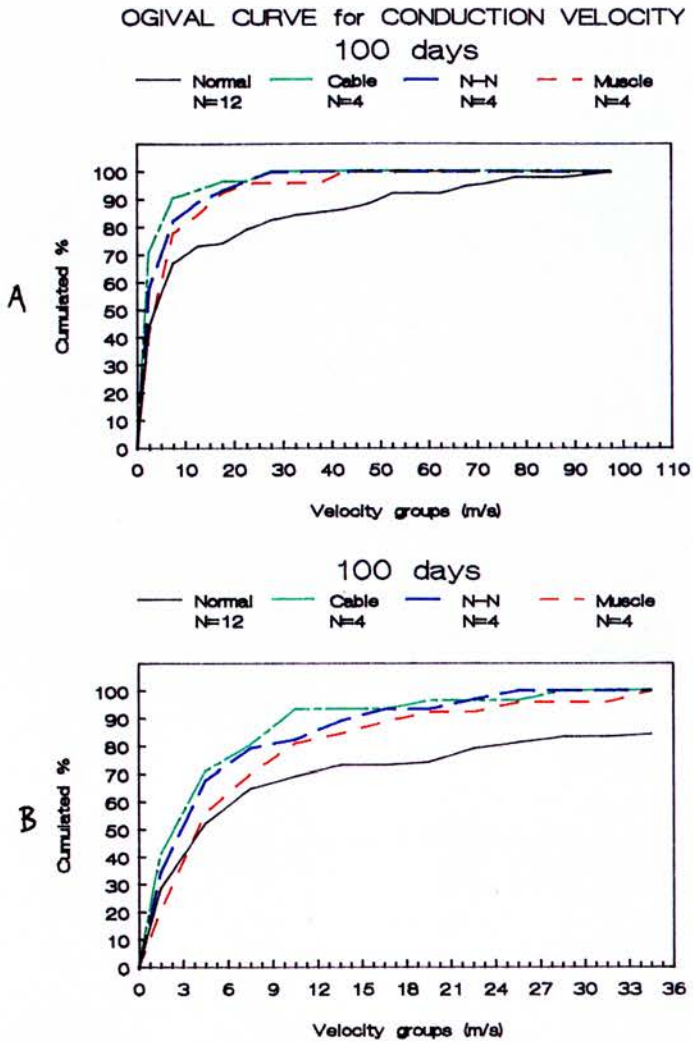


Figure 22: Ogival curves for conduction velocity in normal and repaired sciatic nerves at 100 days.
A: All values. B: Values up to 36 m/sec.

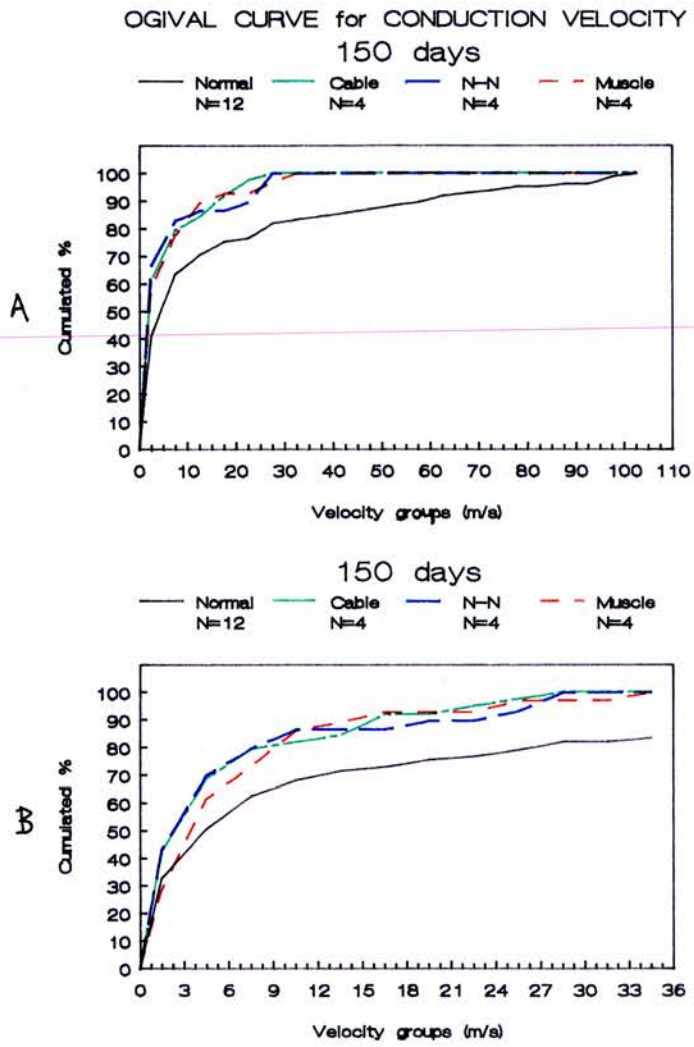


Figure 23: Ogival curves for conduction velocity in normal and repaired sciatic nerves at 150 days.

A: All values. B: Values up to 36 m/sec.

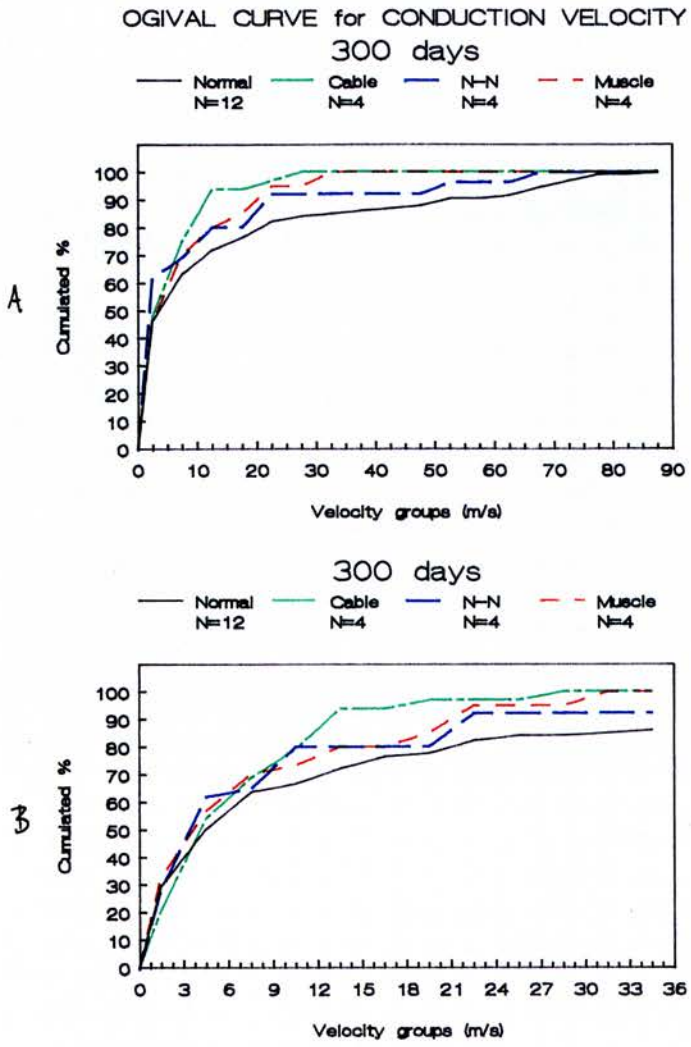


Figure 24: Ogival curves for conduction velocity in normal and repaired sciatic nerves at 300 days.

A: All values. B: Values up to 36 m/sec.

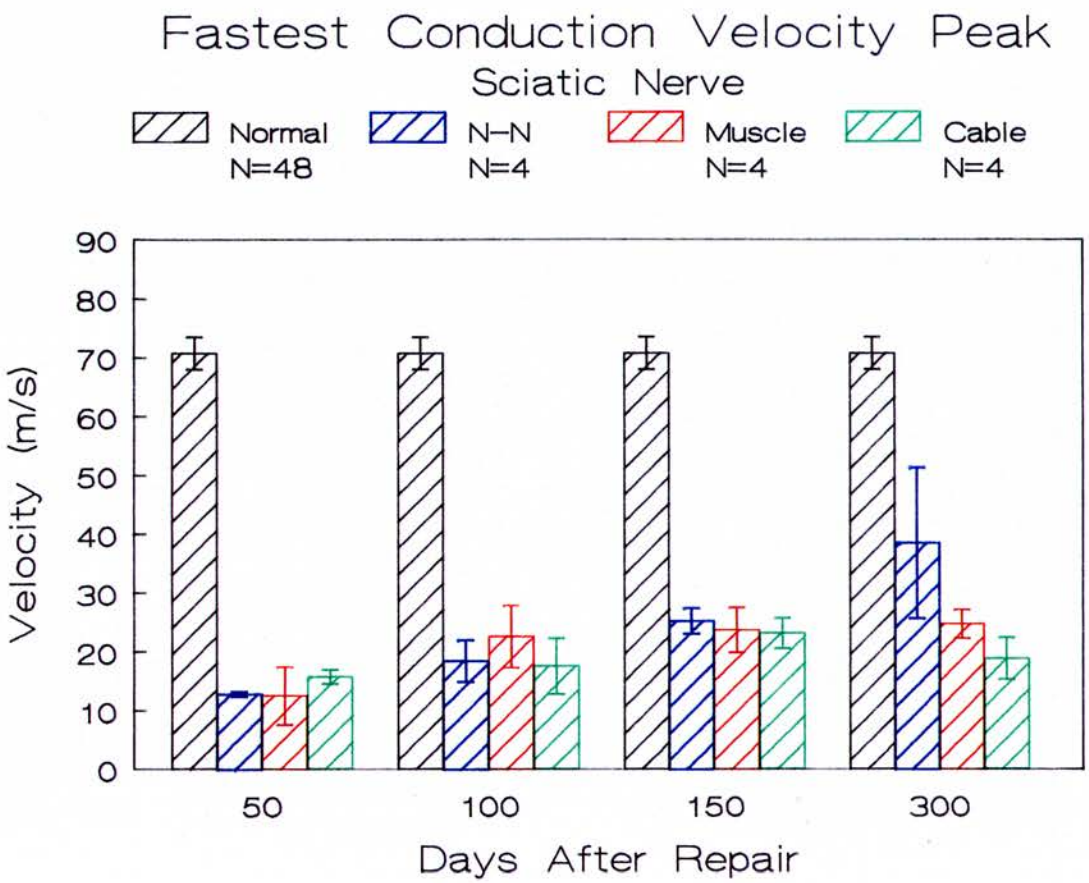


Figure 25: Fastest conduction velocity peak in sciatic nerve. Normal represents pooled data from the unoperated side in all experimental animals.

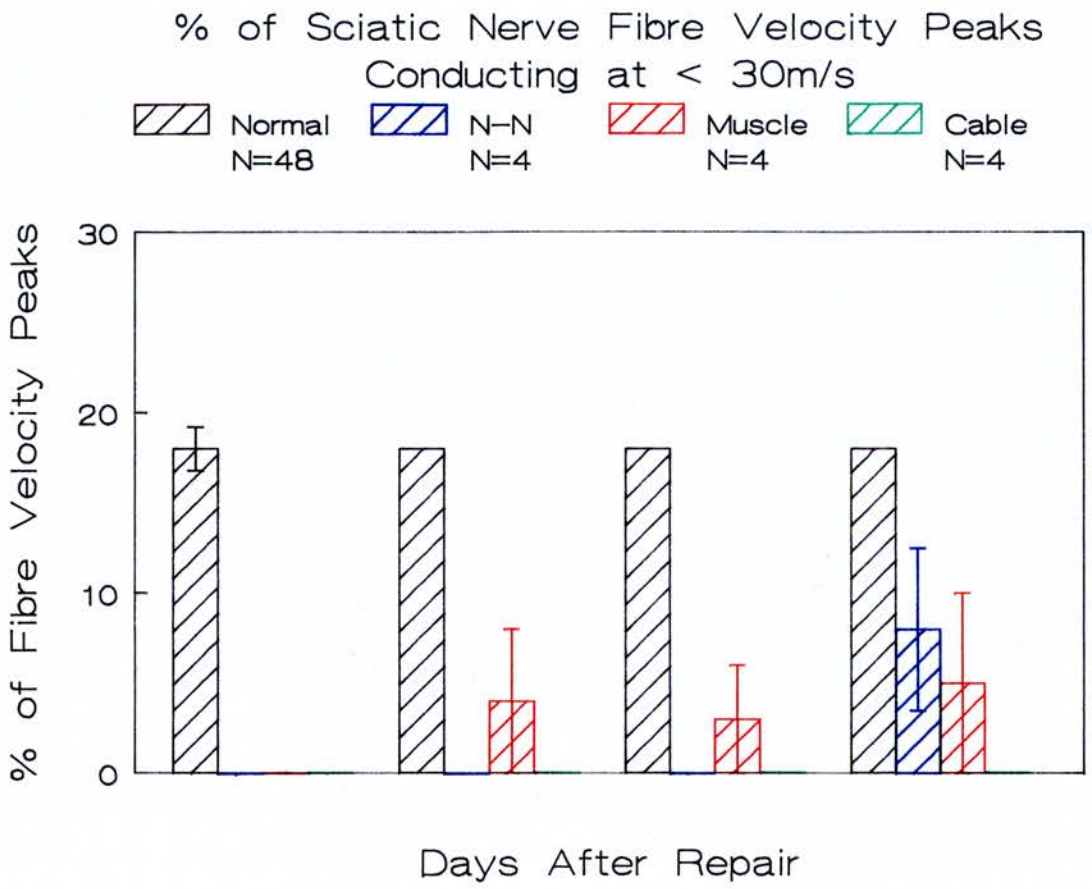
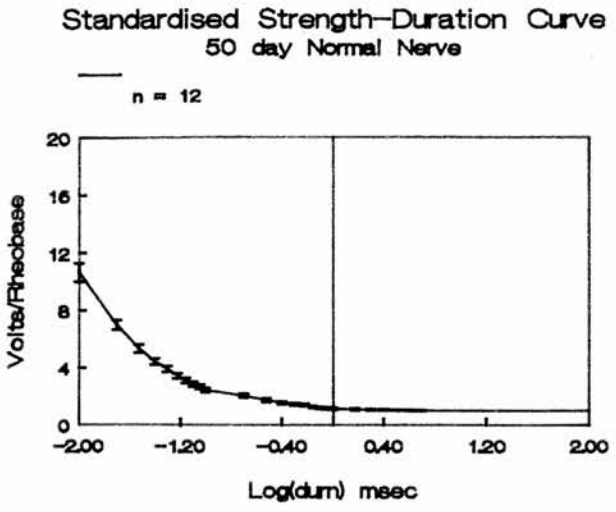
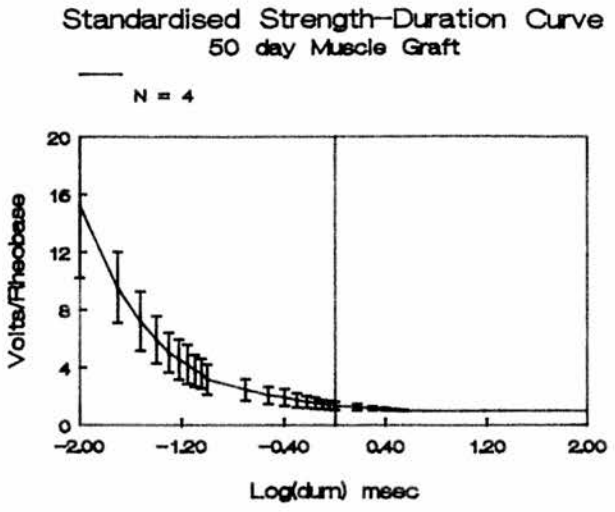
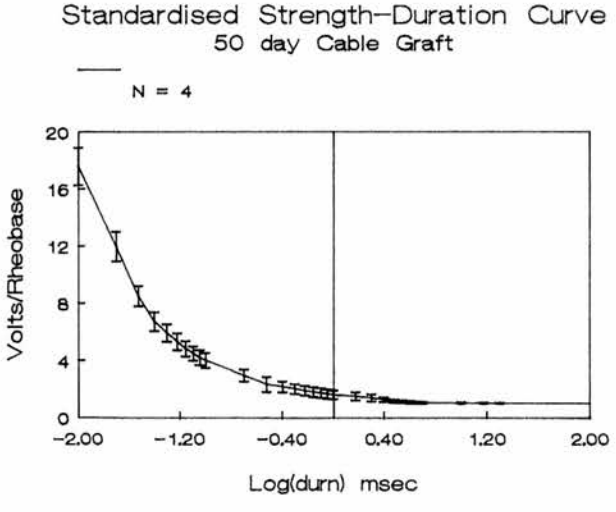


Figure 26: Percentage of sciatic nerve fibre velocity peaks conducting at > 30 m/sec.







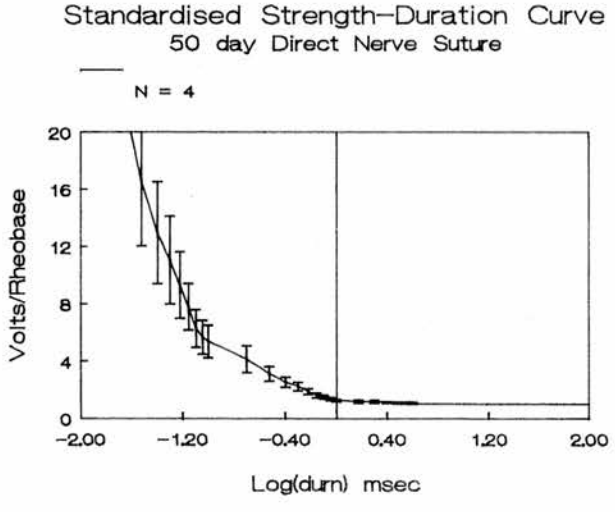
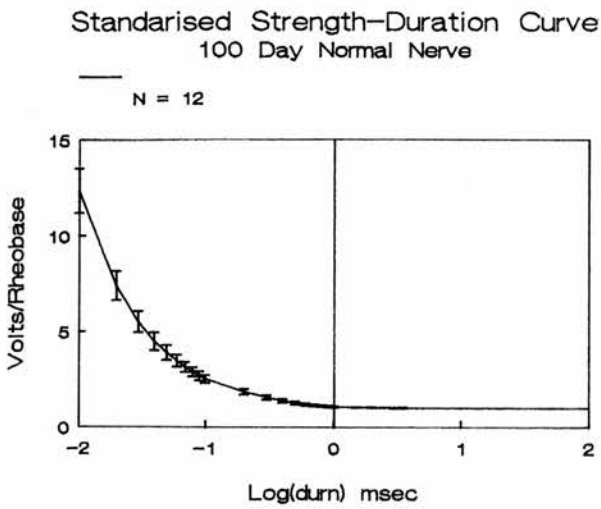
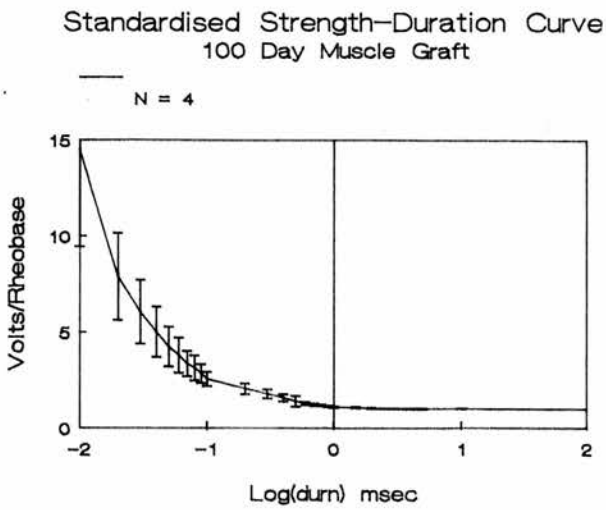
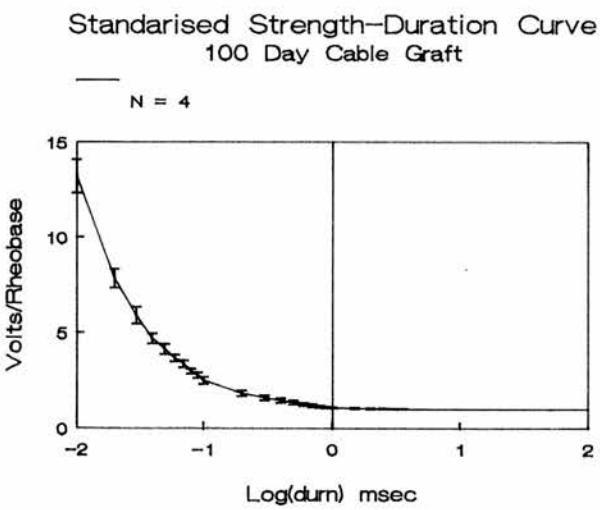


Figure 27: Mean standardised strength-duration curves at 50 days after repair.







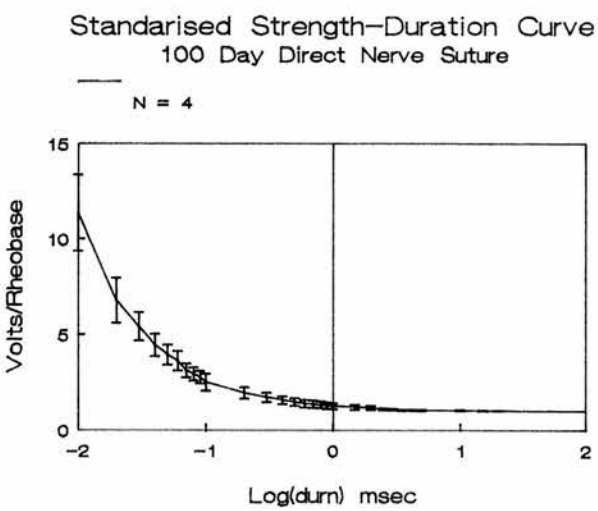
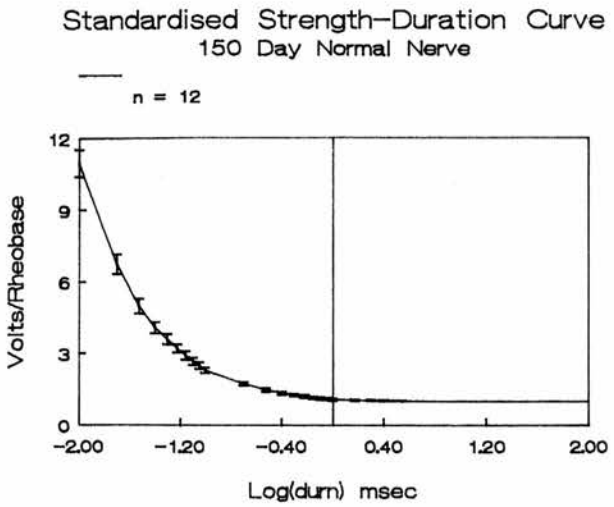
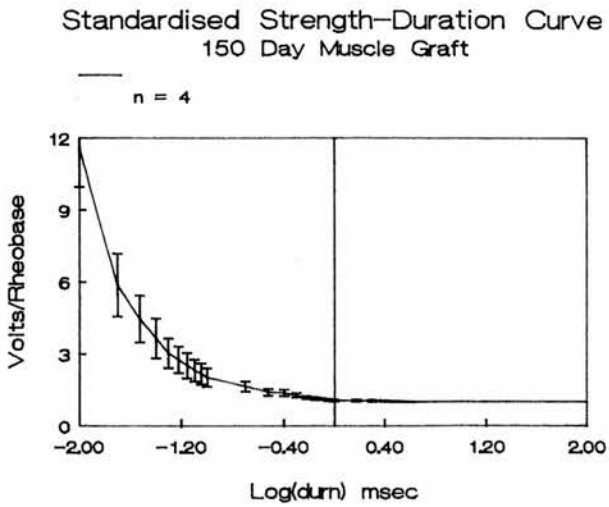
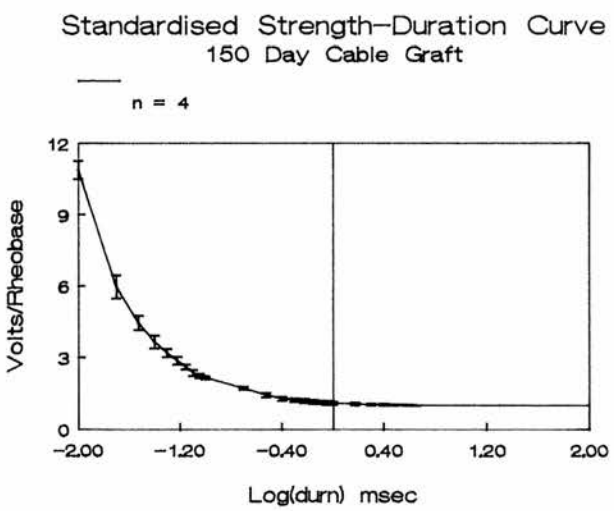


Figure 28: Mean standardised strength-duration curves at 100 days after repair.







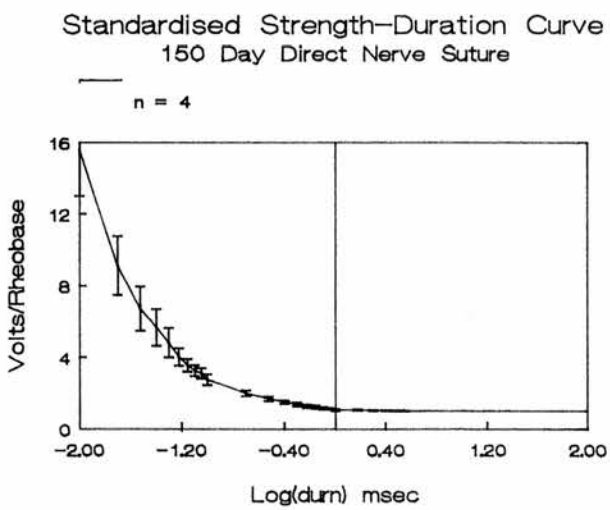
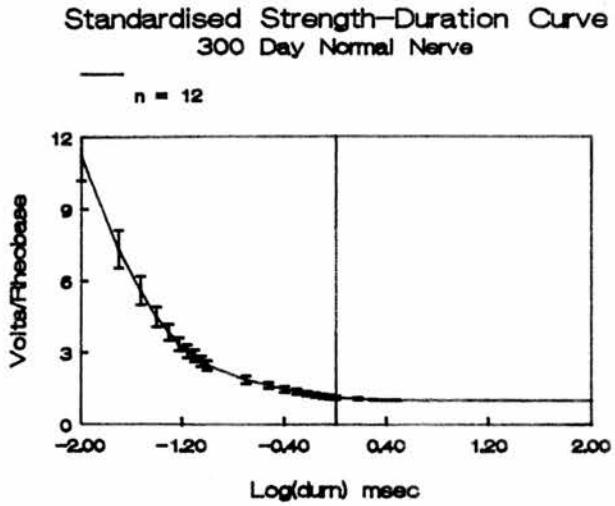
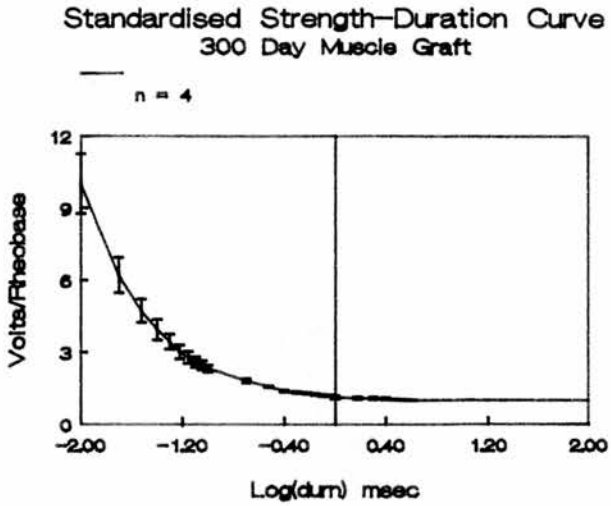
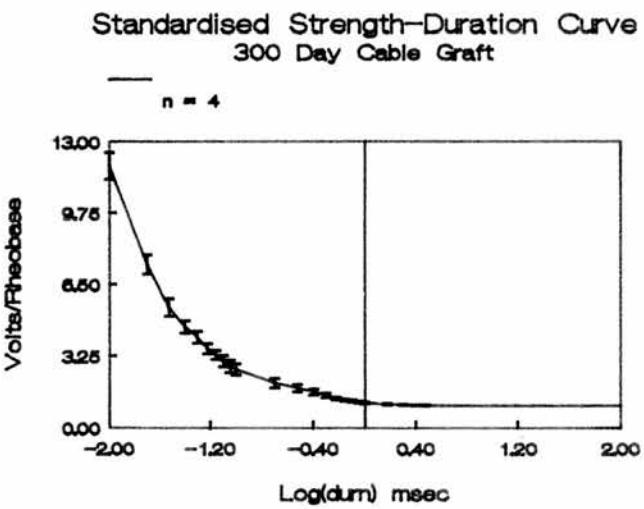


Figure 29: Mean standardised strength-duration curves at 150 days after repair.







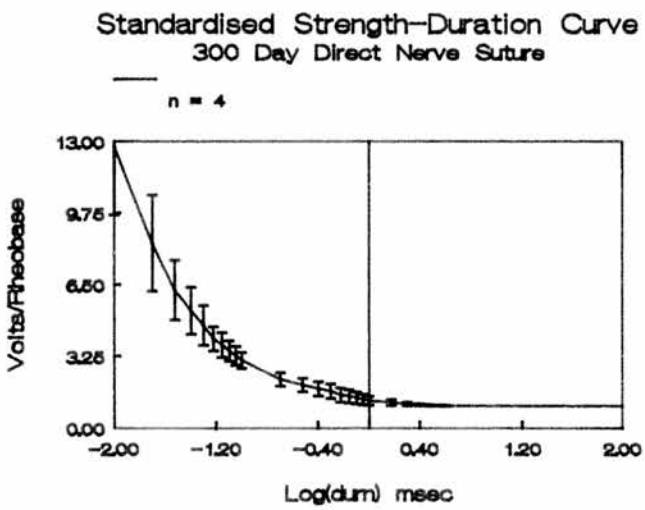
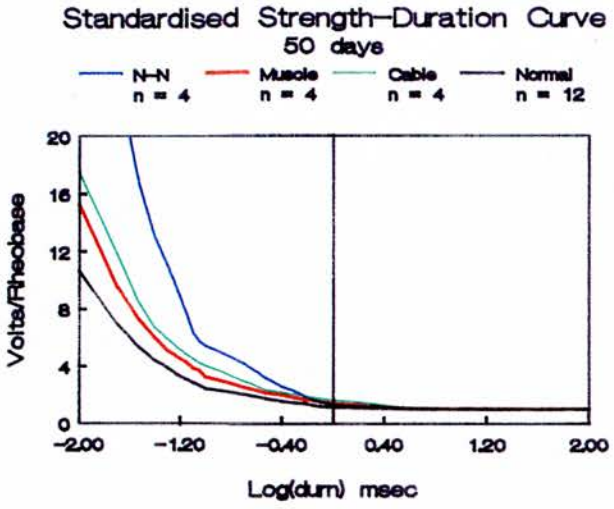
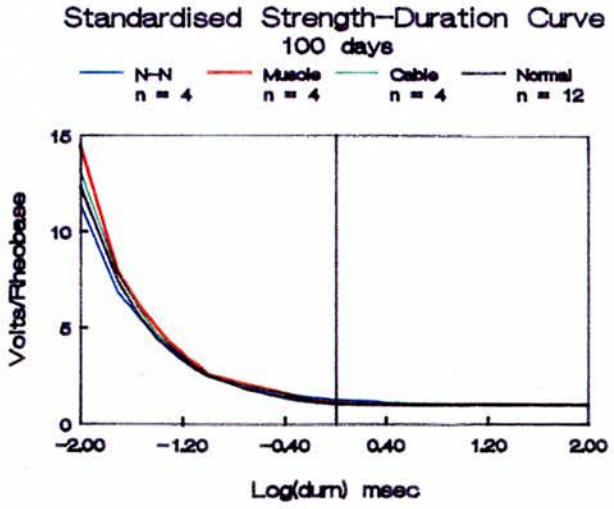
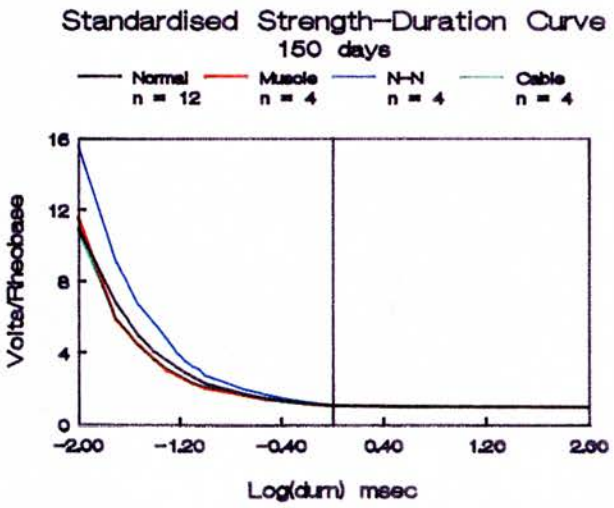


Figure 30: Mean standardised strength-duration curves at 300 days after repair.







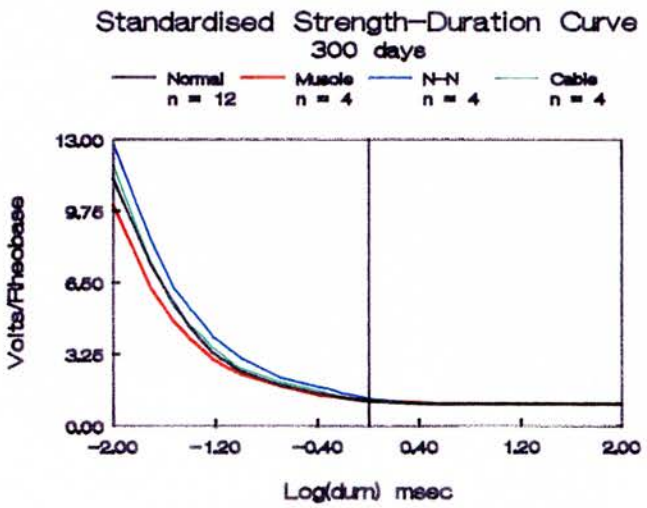


Figure 31: Mean standardised strength-duration curves at 50, 100, 150 and 300 days after repair. (error bars have been omitted for clarity)

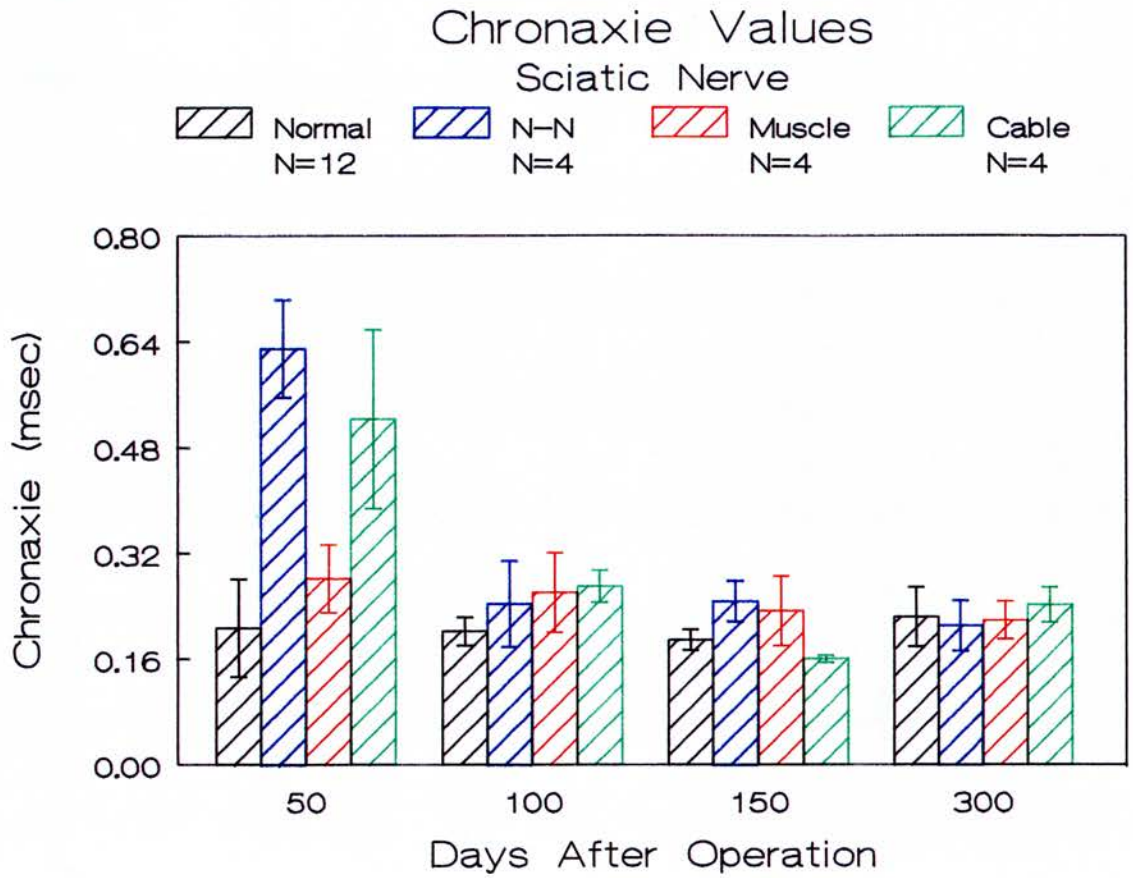


Figure 32: Sciatic nerve chronaxie values. (see text for method of estimation)

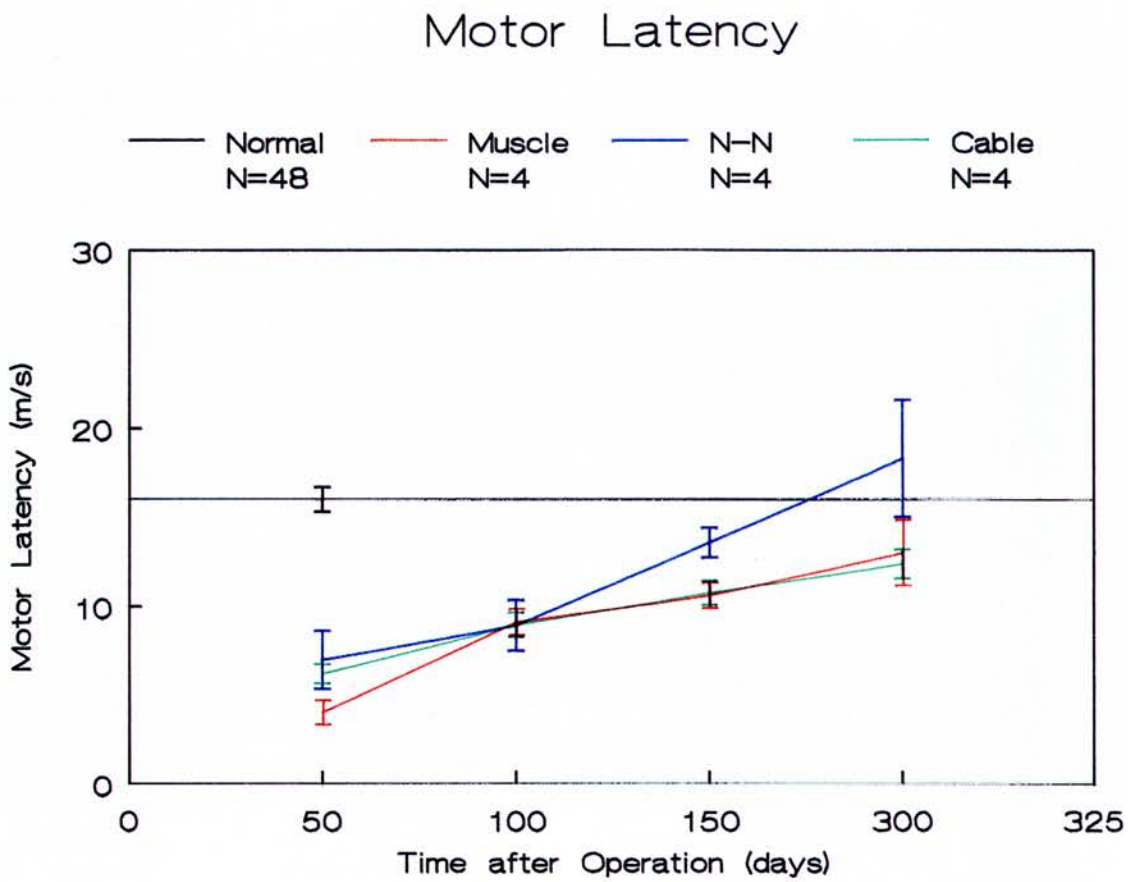


Figure 33: Graph of motor latency values with time after repair of the sciatic nerve, by N-N suture, muscle or cable graft. Normal represents the pooled values of the unoperated side of all experimental animals.

A: 50day Motor Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	p <0.005
Cable	p<0.0005	NS	p <0.005	*

B: 100day Motor Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.005	p<0.005	p<0.005
N–N	p<0.005	*	NS	NS
Muscle	p<0.005	NS	*	NS
Cable	p<0.005	NS	NS	*

C: 150day Motor Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.005	p<0.005
N–N	NS	*	NS	NS
Muscle	p<0.005	NS	*	NS
Cable	p<0.005	NS	NS	*

D: 300day Motor Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

Table 3:7 Motor latency p-values. (NS=p>0.01)

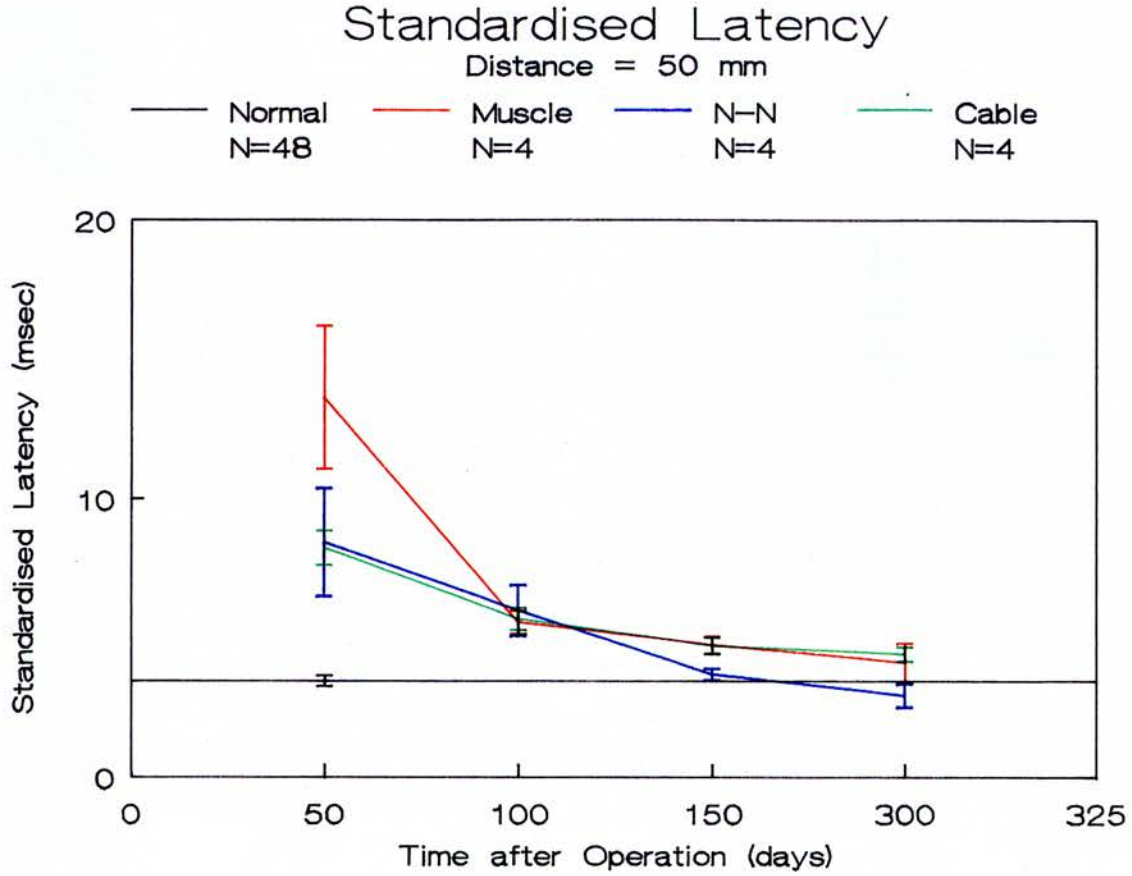


Figure 34: Graph of standardised latency values with time after repair of the sciatic nerve, by N-N suture, muscle or cable graft. Normal represents the pooled values of the unoperated side of all experimental animals.

A: 50day Standardised Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	p < 0.01
Cable	p<0.0005	NS	p < 0.01	*

B: 100day Standardised Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.005	p<0.0025	p<0.0025
N–N	p<0.005	*	NS	NS
Muscle	p<0.0025	NS	*	NS
Cable	p<0.0025	NS	NS	*

C: 150day Standardised Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

D: 300day Standardised Latency P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

Table 3:8 Standardised latency p-values. (NS=p>0.01)

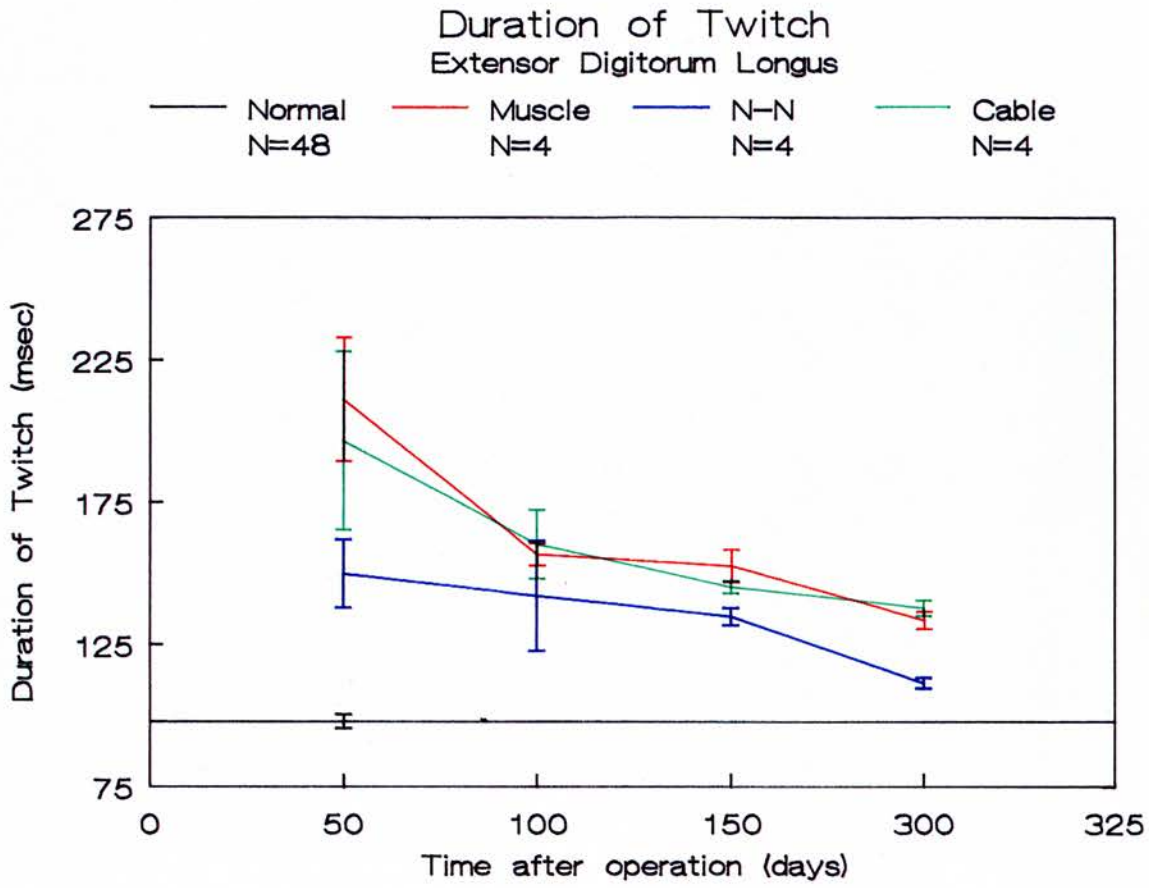


Figure 35: Duration of EDL muscle twitch at various times after sciatic nerve repair, compared to normal.

T = 32°-34°C

A: Duration of Twitch
50day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

B: Duration of Twitch
100day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

C: Duration of Twitch
150day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

D: Duration of Twitch
300day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.0005	p<0.0005
N–N	NS	*	p<0.0025	p<0.0005
Muscle	p<0.0005	p<0.0025	*	NS
Cable	p<0.0005	p<0.0005	NS	*

Table 3:9 Duration of twitch p-values. (NS=p>0.01)

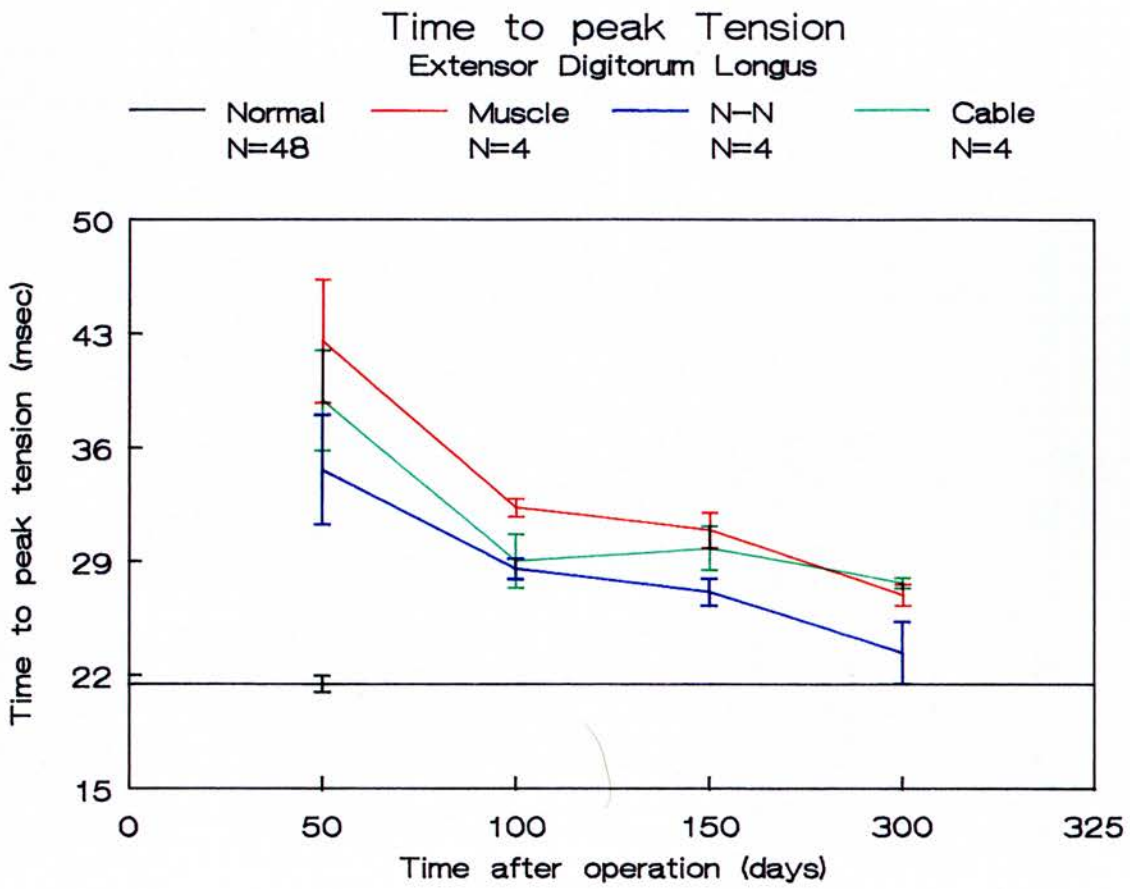


Figure 36: Time to peak isometric tension in EDL at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Time to Peak Tension
50 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0025	p<0.0005	p<0.0005
N–N	p<0.0025	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

B: Time to Peak Tension
100 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p <0.005	p<0.0005	p<0.0005
N–N	p <0.005	*	p <0.005	NS
Muscle	p<0.0005	p <0.005	*	NS
Cable	p<0.0005	NS	NS	*

C: Time to Peak Tension
150 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0025	p<0.0005	p<0.0005
N–N	p<0.0025	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p,0.0005	NS	NS	*

D: Time to Peak Tension
300 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.0025	p<0.0005
N–N	NS	*	NS	NS
Muscle	p<0.0025	NS	*	NS
Cable	p<0.0005	NS	NS	*

Table 3:10 Time to peak twitch p-values. (NS=p>0.01)

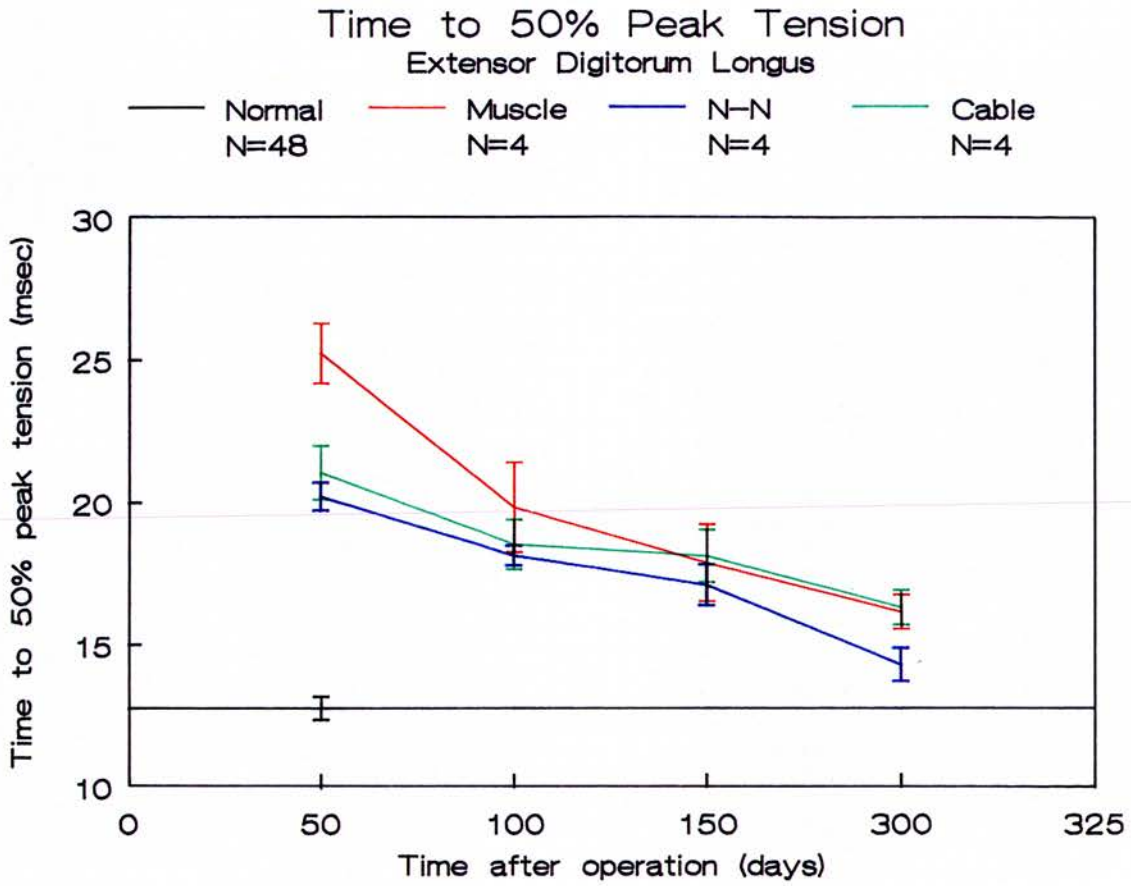


Figure 37: Time to 50% peak isometric tension in EDL at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Time to 50% Peak Tension
50 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	p < 0.01	NS
Muscle	p<0.0005	p < 0.01	*	NS
Cable	p<0.0005	NS	NS	*

B: Time to 50% Peak Tension
100 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

C: Time to 50% Peak Tension
150 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p<0.0025	p<0.0025	p<0.0005
N–N	p<0.0025	*	NS	NS
Muscle	p<0.0025	NS	*	NS
Cable	p<0.0005	NS	NS	*

D: Time to 50% Peak Tension
300 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p < 0.01	p < 0.01
N–N	NS	*	NS	NS
Muscle	p < 0.01	NS	*	NS
Cable	p < 0.01	NS	NS	*

Table 3:11 Time to 50% peak twitch p-values. (NS=p>0.01)

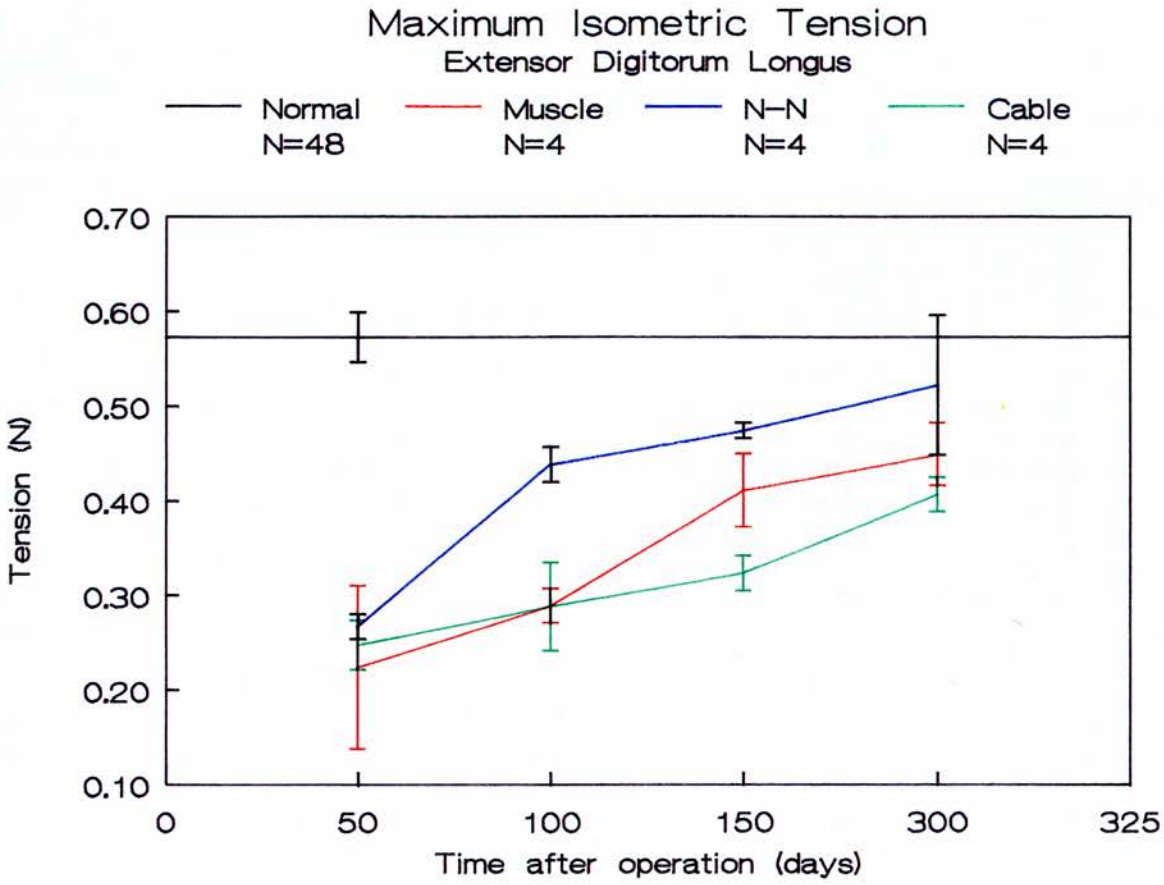


Figure 38: Maximum isometric tension in EDL muscle at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Maximum Isometric Tension
50 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p <0.0025	p<0.0005	p<0.0005
N–N	p <0.0025	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

B: Maximum Isometric Tension
100 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p <0.01	p<0.0025
N–N	NS	*	NS	NS
Muscle	p <0.01	NS	*	NS
Cable	p<0.0025	NS	NS	*

C: Maximum Isometric Tension
150 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	p <0.005
N–N	NS	*	NS	p<0.0005
Muscle	NS	NS	*	NS
Cable	p <0.005	p<0.0005	NS	*

D: Maximum Isometric Tension
300 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

Table 3:12 Maximum twitch p-values. (NS=p>0.01)

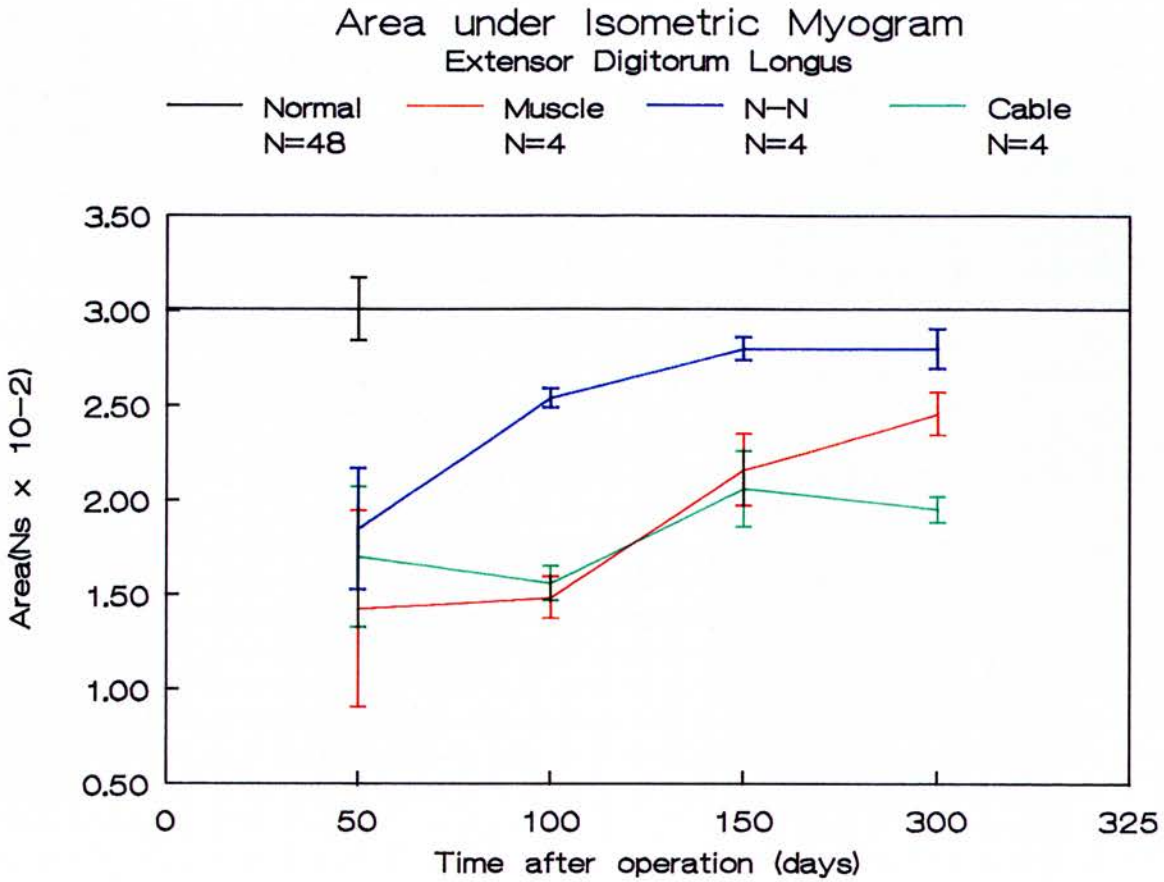


Figure 39: Area under the isometric myogram (time-tension integral) at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Area Under Isometric Myogram
50day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p < 0.01	p < 0.01	p < 0.01
N–N	p < 0.01	*	NS	NS
Muscle	p < 0.01	NS	*	NS
Cable	p < 0.01	NS	NS	*

B: Area Under Isometric Myogram
100day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p <0.01	p <0.01
N–N	NS	*	p<0.0005	p<0.0005
Muscle	p <0.01	p<0.0005	*	NS
Cable	p <0.01	p<0.0005	NS	*

C: Area Under Isometric Myogram
150day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	p < 0.01
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	p < 0.01	NS	NS	*

D: Area Under Isometric Myogram
300day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	p<0.0005
Muscle	NS	NS	*	p < 0.01
Cable	NS	p<0.0005	p < 0.01	*

Table 3:13 Area under the isometric myogram (time-tension integral) p-values. (NS=p>0.01)

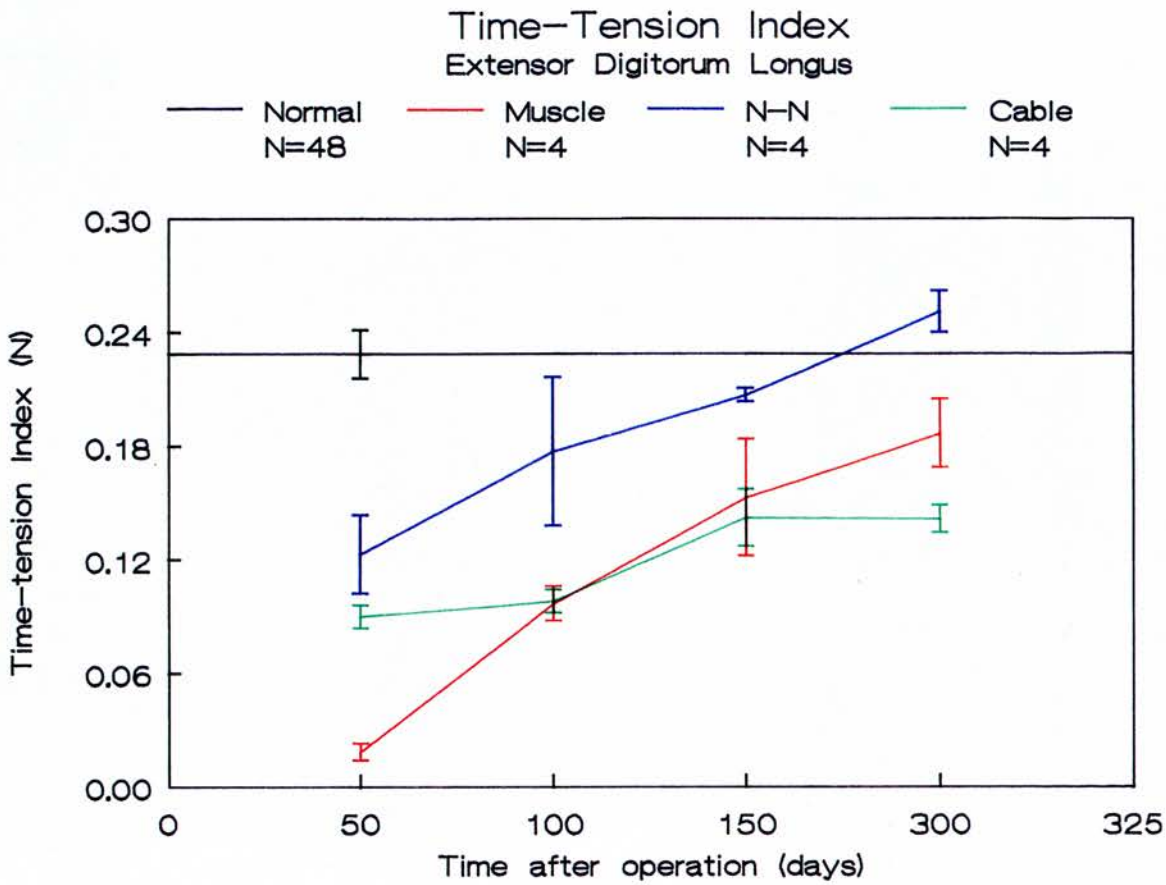


Figure 40: "Average" tension (time-tension index) at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Time–Tension Index
50 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.0005	p<0.0025
N–N	NS	*	p<0.005	NS
Muscle	p<0.0005	p<0.005	*	p<0.0005
Cable	p<0.0025	NS	p<0.0005	*

B: Time–Tension Index
100day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p<0.005	p<0.005
N–N	NS	*	NS	NS
Muscle	p<0.005	NS	*	NS
Cable	p<0.005	NS	NS	*

C: Time–Tension Index
150day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	p<0.005	p < 0.01
Muscle	NS	p<0.005	*	NS
Cable	NS	p <0.01	NS	*

D: Time–Tension Index
300day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	p<0.0005
Muscle	NS	NS	*	NS
Cable	NS	p<0.0005	NS	*

Table 3:14 "Average" tension (time-tension index) p-values. (NS=p>0.01)

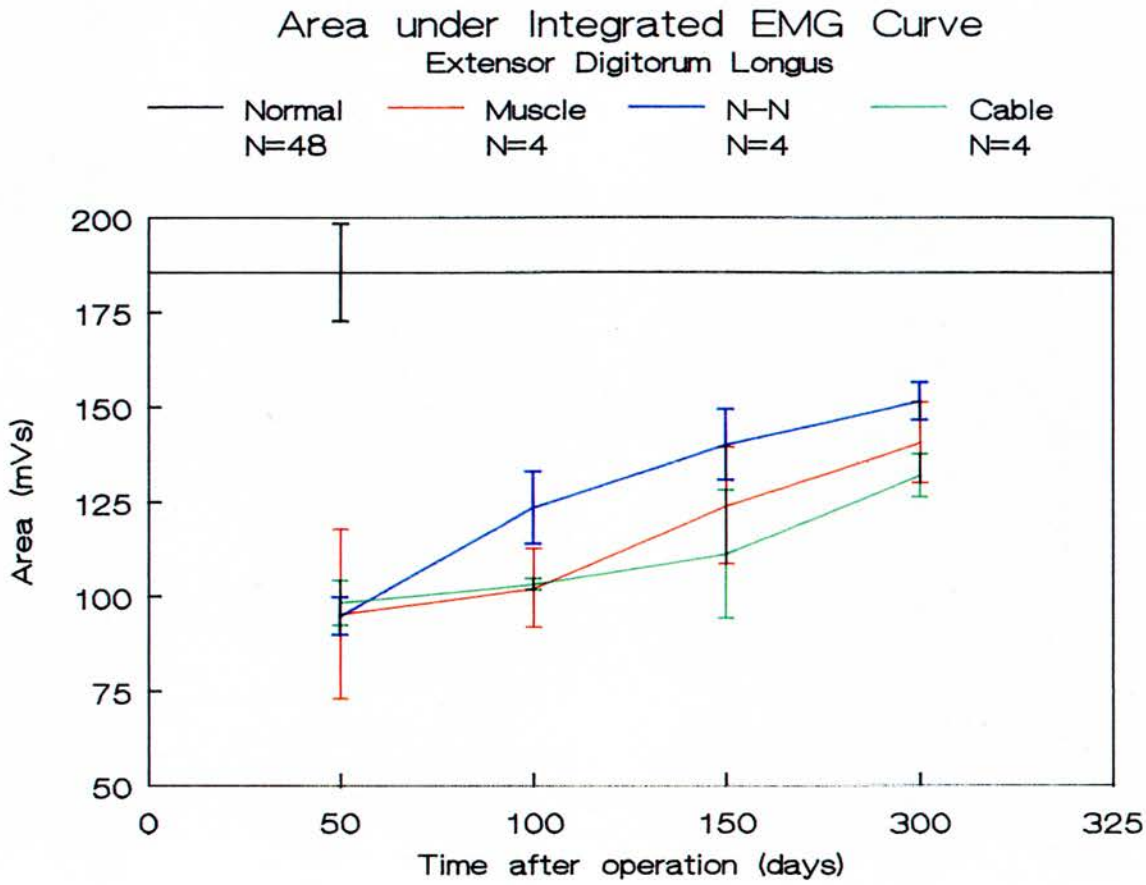


Figure 41: Area under the integrated EMG curve at various times after sciatic nerve repair, compared to normal.

A: Area Under the Integrated EMG
50 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	p < 0.01	NS	p < 0.01
N–N	p < 0.01	*	NS	NS
Muscle	NS	NS	*	NS
Cable	p < 0.01	NS	NS	*

B: Area Under the Integrated EMG
100 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	p < 0.01	p < 0.01
N–N	NS	*	NS	NS
Muscle	p < 0.01	NS	*	NS
Cable	p < 0.01	NS	NS	*

C: Area Under the Integrated EMG
150 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

D: Area Under the Integrated EMG
300 day P Values

	Normal	N–N	Muscle	Cable
Normal	*	NS	NS	NS
N–N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

Table 3:15 Area under the integrated EMG p-values.
(NS=p>0.01)

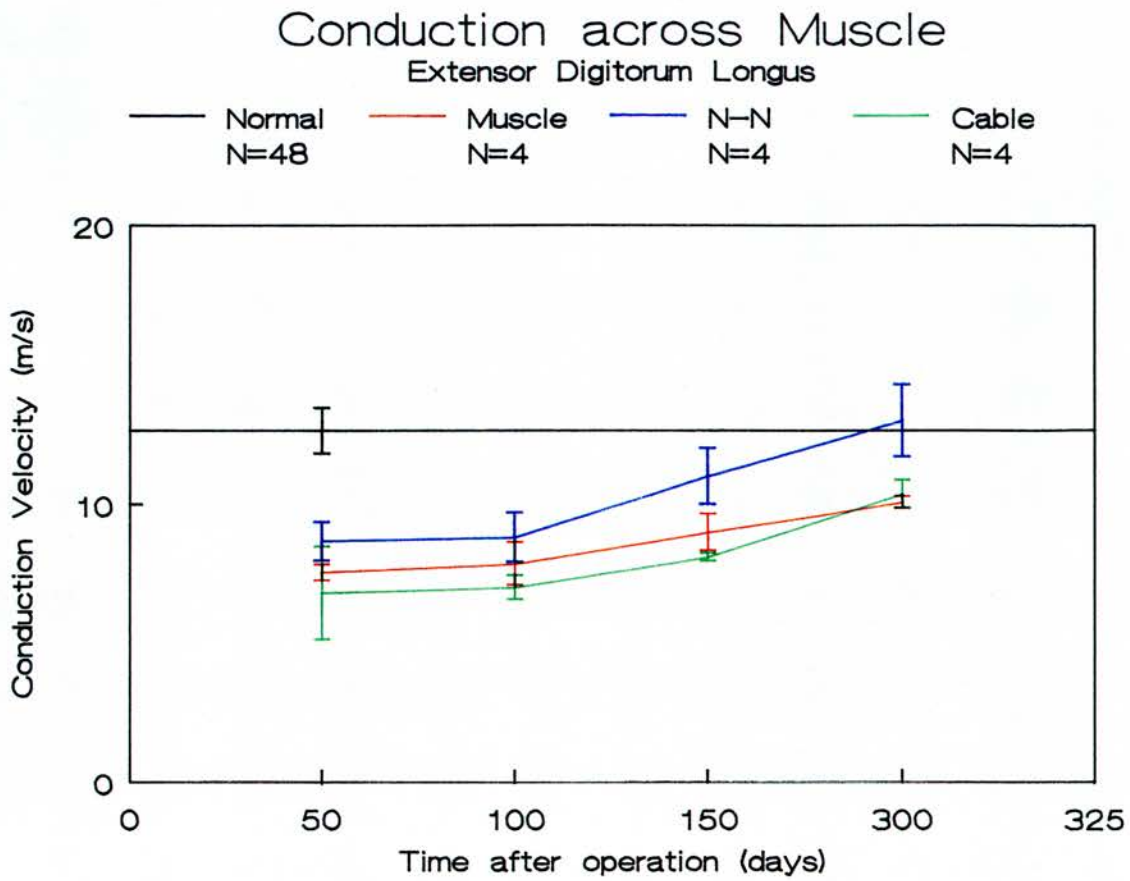


Figure 42: Conduction across isolated EDL muscle at various times after sciatic nerve repair, compared to normal. T = 32°-34°C

A: Conduction Across Muscle
50day P Values

	Normal	N-N	Muscle	Cable
Normal	*	NS	NS	NS
N-N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

B: Conduction Across Muscle
100day P Values

	Normal	N-N	Muscle	Cable
Normal	*	NS	NS	NS
N-N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

C: Conduction Across Muscle
150day P Values

	Normal	N-N	Muscle	Cable
Normal	*	NS	NS	NS
N-N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

D: Conduction Across Muscle
300day P Values

	Normal	N-N	Muscle	Cable
Normal	*	NS	NS	NS
N-N	NS	*	NS	NS
Muscle	NS	NS	*	NS
Cable	NS	NS	NS	*

Table 3:16 Conduction across isolated EDL muscle p-values. (NS=p>0.01)

Muscle Weights (g)

	Normal	Muscle	N–N	Cable
50 days	N=12 x=0.2191 SD=0.0275	N=4 x=0.0799 SD=0.0027	N=4 x=0.1300 SD=0.0137	N=4 x=0.1066 SD=0.0060
100 days	N=12 x=0.2448 SD=0.0320	N=4 x=0.1272 SD=0.0082	N=4 x=0.1980 SD=0.0441	N=4 x=0.1311 SD=0.0089
150 days	N=12 x=0.2441 SD=0.0185	N=4 x=0.1899 SD=0.0167	N=4 x=0.2129 SD=0.0144	N=4 x=0.1819 SD=0.0078
300 days	N=12 x=0.2540 SD=0.0247	N=4 x=0.2180 SD=0.0214	N=4 x=0.2407 SD=0.0079	N=4 x=0.2200 SD=0.0194

Table 3:17 EDL muscle weights and statistics.

A: 50day Muscle Weight P Values

	Normal	N–N	Muscle	Cable
Normal	✳	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	✳	p<0.0025	NS
Muscle	p<0.0005	p<0.0025	✳	p<0.0025
Cable	p<0.0005	NS	p<0.0025	✳

B: 100day Muscle Weight P Values

	Normal	N–N	Muscle	Cable
Normal	✳	p<0.005	p<0.0005	p<0.0005
N–N	p<0.005	✳	NS	NS
Muscle	p<0.0005	NS	✳	NS
Cable	p<0.0005	NS	NS	✳

C: 150day Muscle Weight P Values

	Normal	N–N	Muscle	Cable
Normal	✳	p < 0.01	p<0.0005	p<0.0005
N–N	p < 0.01	✳	NS	NS
Muscle	p<0.0005	NS	✳	NS
Cable	p<0.0005	NS	NS	✳

D: 300day Muscle Weight P Values

	Normal	N–N	Muscle	Cable
Normal	✳	NS	NS	NS
N–N	NS	✳	NS	NS
Muscle	NS	NS	✳	NS
Cable	NS	NS	NS	✳

Table 3:18 EDL muscle weights p-values. (NS=p>0.01)

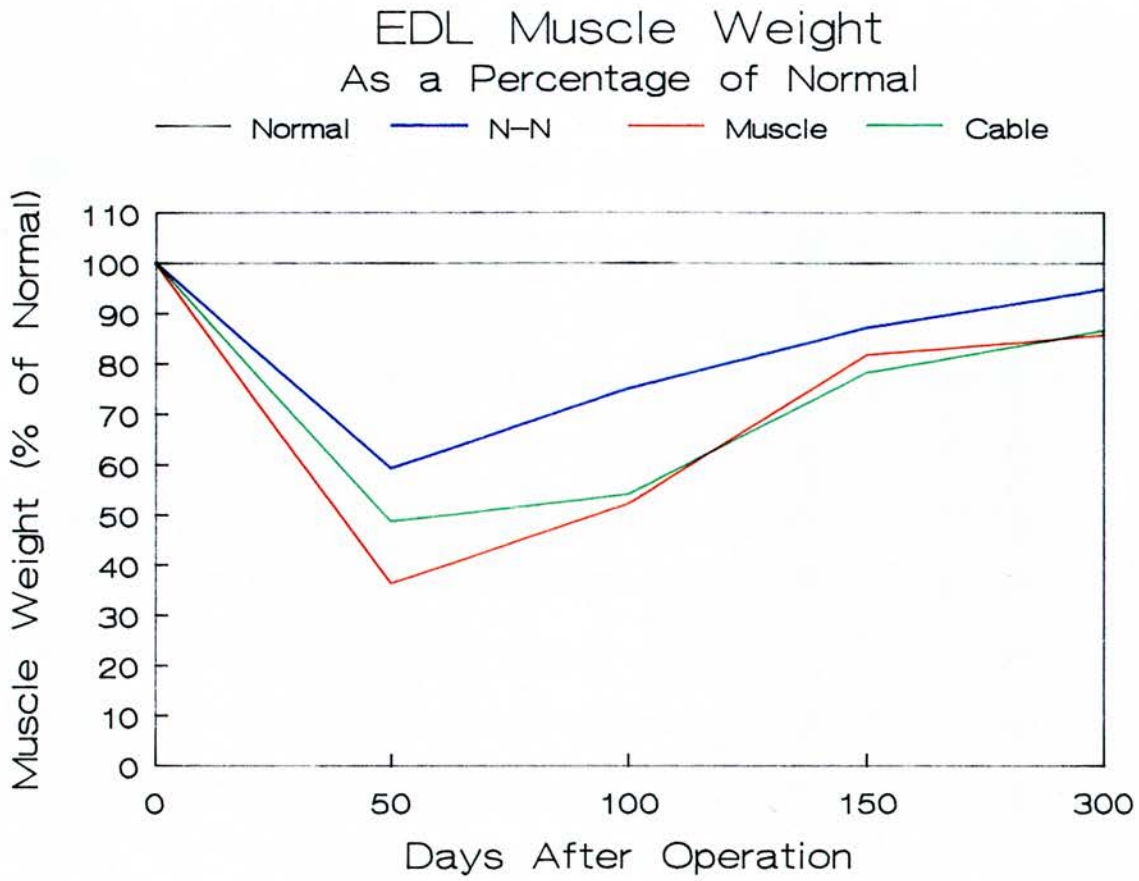


Figure 43: EDL muscle weights as a percentage of normal. (normal = 100%)

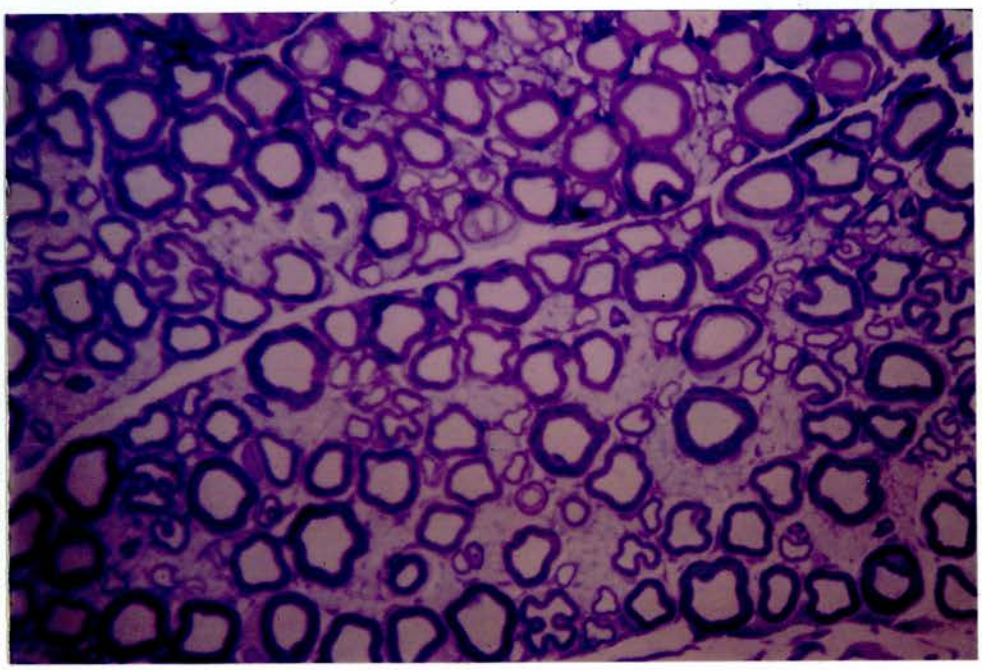


Figure 44: Semithin, plastic embedded Toluidine Blue section of normal proximal sciatic nerve. x914
Note the abundance of large diameter fibres with well developed myelin sheaths.

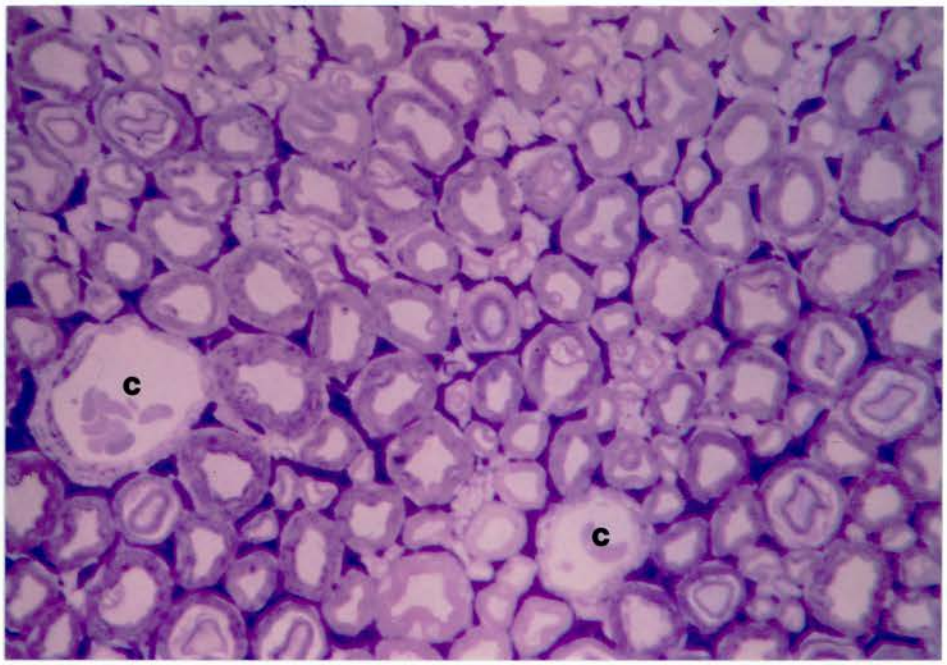


Figure 45: Semithin, plastic embedded Toluidine-Blue section of normal peroneal nerve. x914

c = intraneural capillary

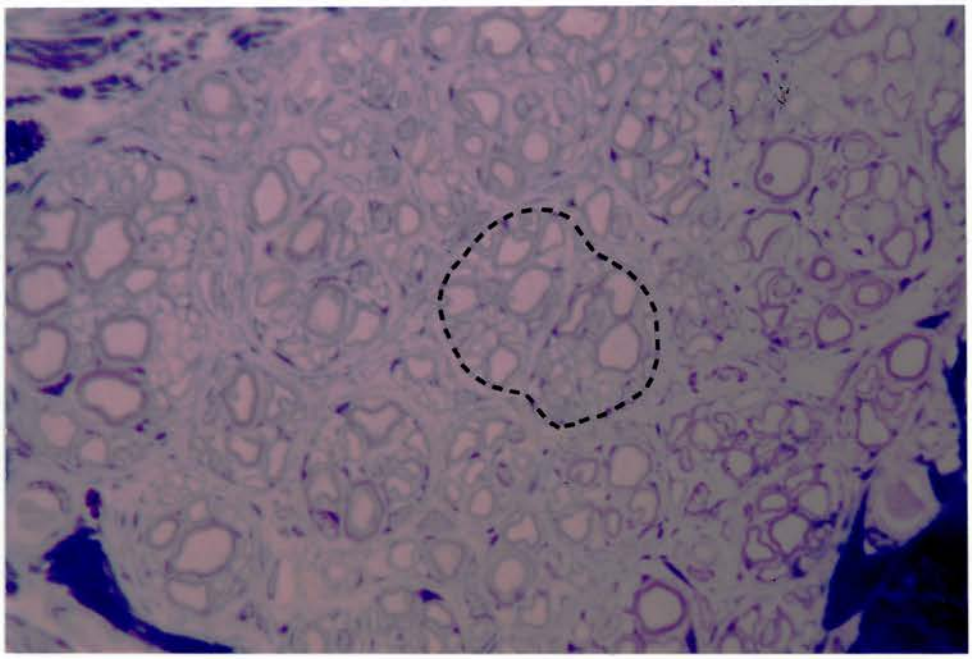


Figure 46: Semithin, plastic embedded Toluidine Blue section through a muscle graft at 300 days. x914
The pattern of the muscle fibre BM can still be seen.

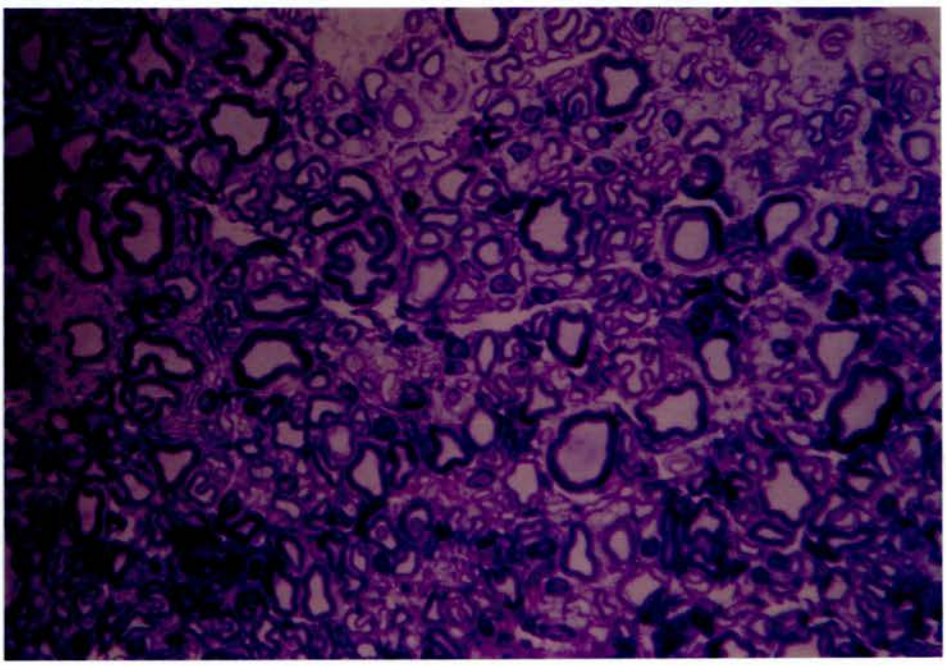


Figure 47: Semithin, plastic embedded Toluidine Blue section through a cable graft at 300 days. x914

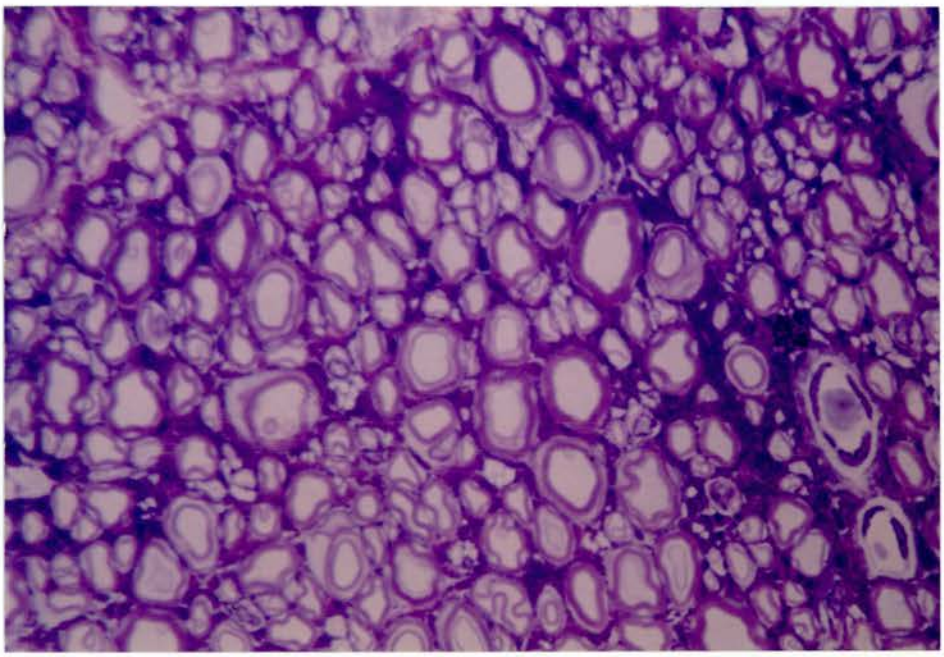


Figure 48: Semithin, plastic embedded Toluidine Blue section of peroneal nerve, 300 days after repair by muscle graft. x914

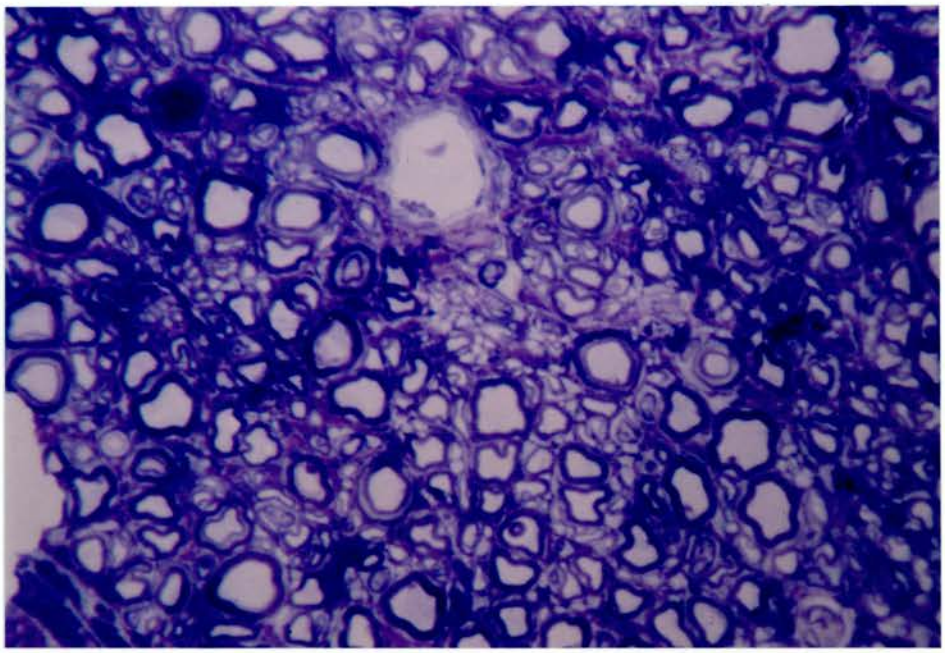


Figure 49: Semithin, plastic embedded Toluidine Blue section of peroneal nerve, 300 days after repair by three-strand cable graft. x914

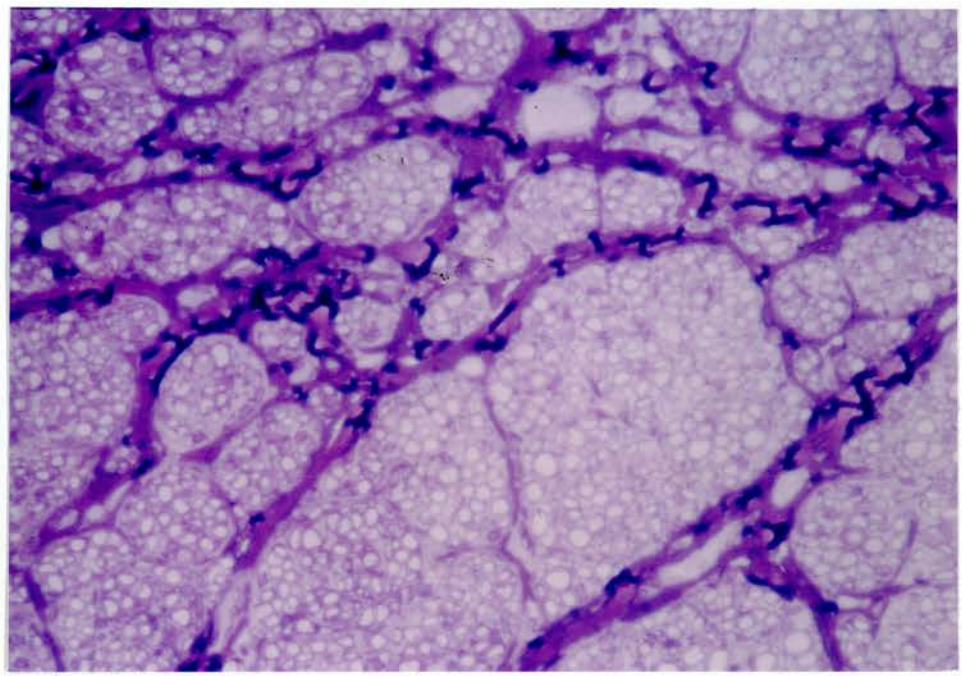


Figure 50: Semithin, plastic embedded Toluidine Blue section of muscle graft, showing ingrowth of large numbers of axon sprouts (50 days) x430

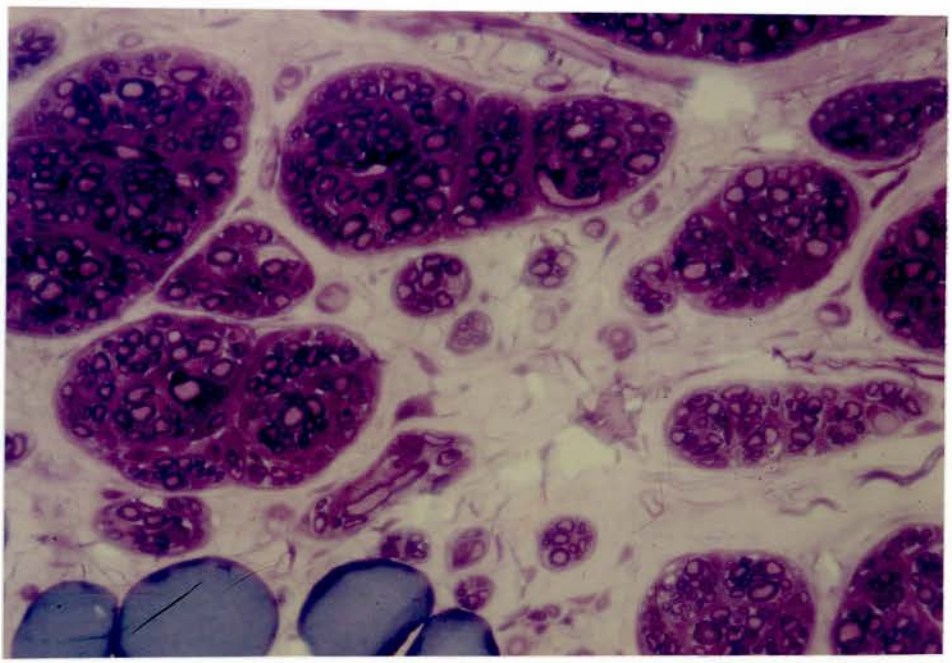


Figure 51: Semithin, plastic embedded Toluidine Blue section of muscle graft (100 days) x430. Myelination has begun.

3:4 Discussion

3:4:1 Fibre counts.

Fibre counts can be very misleading after nerve regeneration owing to excessive axon sprouting. This is shown well in figures 17 and 20. Fibres in excess of normal do not necessarily imply a better result since presumably, the fewer fibres in the normal nerve were able to give full function. Indeed it has been shown, in the rabbit, that when regeneration was almost perfect (after axonotmesis) fibre numbers attained normal values (Gutmann and Sanders, 1943). Aird and Naffziger (1939), found that after division of the peroneal and tibial fascicles of the sciatic nerve, with suture of the proximal peroneal stump to both distal tibial and peroneal stumps, fibre numbers in the distal nerves were increased by one third after eight and a half months. They also noted that this was associated with a loss of muscle bulk, presumably due to denervation atrophy. However, Gutmann and Sanders (1943), found a decrease in fibre numbers to 87% of normal, distal to a direct nerve suture in the rabbit. The same authors found an increase in fibre numbers to 105% within a full thickness autologous nerve graft (at 200 days) but a decrease to 79% in the distal stump at the same time.

Mackinnon and Trued (1986) found a much increased number of myelinated axons both in the graft (173% approx.) and in the distal stump (133% approx.) at six months after a full thickness autologous nerve graft in the rat sciatic

nerve.

Figure 20 shows an increase in the number of fibres within the muscle graft to 118% and an increase in the cable graft to 121% at 300 days, when compared to proximal sciatic nerve. The distal stump (peroneal nerve in this case) showed an increase to 108% after N-N suture, 127% after muscle graft and 122% after cable graft when compared to normal peroneal nerve at 300 days (Figure 17). It would seem from the above accounts (and many others) that the absolute number of axons after nerve repair can be very variable and may not reflect the degree of functional recovery. Obviously when nerve fibre counts are poor this can be taken to imply poor regeneration but, as in this case, when abundant sprouting has taken place, to supranormal levels, it can be misleading to make comparisons between various methods of repair based on fibre counts alone. This is especially true when different methods of counting are employed. It was for this reason that indices of fibre maturation were also taken into account in this study.

3:4:2 Fibre Diameters and Myelination

Maturation can be divided into two processes:

a) attainment of normal fibre diameter and
b) maturation of the myelin sheath.

Nerve fibres of different sizes (diameters) are represented in the fibre population of a peripheral nerve in definite frequencies. This frequency distribution, known as the fibre diameter spectrum, varied from nerve

to nerve but was relatively constant for any given nerve under normal conditions (Weiss, Edds and Cavanaugh, 1945). After injury and regeneration the fibre diameter was changed. How closely the restored spectrum resembled the normal depended on the degree of completeness of regeneration. The fibre spectrum was restored almost to normal after a crush injury (axonotmesis) (Gutmann and Sanders 1943), was significantly shifted to smaller diameters after nerve suture (Gutmann and Sanders, 1943; Sanders and Young, 1944; Hammond and Hinsey, 1945; Aitken and Thomas, 1962) but was most abnormal, with much smaller diameter fibres, after nerve grafting (Gutmann and Sanders, 1943; Mackinnon and Trued, 1986). It has been suggested that fibre diameter depends on the formation of appropriate peripheral connections (Weiss, Edds and Cavanaugh, 1945; Aitken, Sharman and Young, 1947; Sanders and Young, 1947; Aitken and Thomas, 1962) and also on the size of the graft and distal stump endoneurial tubes (Sanders and Young, 1944; Hammond and Hinsey, 1945). Thus, even if fibre numbers were the same, if the fibre spectra were different for two methods of repair then conclusions could be drawn about the degree of disorganisation of regenerating fibres.

Figure 18 showed that, as previously found, the fibre diameter spectrum was shifted to the left after all types of repair and that this was significantly worse after grafting methods than after direct nerve suture. This presumably reflects increased disorganisation of axon sprouts when regenerated through a graft. Interestingly

there was no significant difference between the mean fibre diameter found in cable or muscle grafted nerves. The normal peroneal nerve displayed a bimodal pattern of fibre diameters as found previously in various rabbit motor nerves (Gutmann and Sanders, 1943; Fernand and Young, 1951). It is the faster of the two peaks that is lost after regeneration, these being A-alpha and A-beta motor fibres and type Ia, Ib and II sensory fibres from muscle spindles and tendon organs (Tables 3:1 and 4:1). Table 3:5 shows fibre diameter values in the graft. Again there were no significant differences between muscle and cable grafted nerves, suggesting that neither graft material had a constricting effect on growing nerve fibres.

Myelin sheath thickness in the distal stump was significantly reduced in all nerves 300 days after repair. Muscle and cable grafted nerves were not different from each other in this respect. Myelination in the muscle graft was not significantly different from that in the cable graft. However the degree of myelination in the muscle graft was significantly less than the degree of myelination in its own distal nerve. A delay in myelination has been reported previously in muscle grafts (Gattuso, Glasby & Gschmeissner, 1988) when compared to their own distal stumps and probably reflects the time taken for Schwann cell ingrowth into the muscle graft. It would be interesting to look at very long grafts to see if myelination is more retarded in the centre of these grafts than at the periphery. Nadime,

Anderson and Turmaine (1990) have looked at this problem in long freeze-dried nerve grafts in the rat and have concluded that axonal regeneration is limited through long grafts due to the limited migratory powers of Schwann cells from the proximal stump. Unfortunately these authors did not take into account the greater migratory response of Schwann cells from the distal stump or the chemotactic influences associated with the products of the distal stump. Even so, they remain doubtful of the ability of freeze-thawed nerve grafts to sustain nerve regeneration over distances of greater than 40mm. It has been shown that axons will regenerate through a 12cm freeze-thawed muscle graft placed in the common peroneal nerve in a patient, following a gunshot wound to the leg. Muscle function was seen to return by 5 months (Mr Rolphe Birch, Consultant Orthopaedic Surgeon, St. Mary's Hospital, London, personal communication, 1990.) This is a significant length of graft in the human. It would seem that the conclusions drawn by Nadime, Anderson and Turmaine do not apply to the muscle graft attached to a peripheral stump.

It must be noted that the cable grafts used in this study were "gold-standard" grafts in that each cable strand was made from a mixed nerve containing large diameter endoneurial tubes. This would not be the case in the clinical situation where small sensory nerves have to be used, thus furnishing grafts with smaller diameter tubes. The muscle graft results are encouraging in another respect. Individual cable strands were aligned with the

peroneal fascicle and the tibial fascicles in an attempt to guide axons from the proximal stump into the appropriate fascicle in the distal stump. This is a time consuming business when there are many fascicles in a nerve but is thought to increase the numbers of appropriate connections. Obviously it is impossible to align a muscle graft so precisely. It is interesting that this seemed to have no effect on the eventual outcome in this case.

3:4:3 Conduction Velocity and Excitability.

In a mixed peripheral nerve, different groups of fibres, characterised by different diameters and degrees of myelination, are responsible for different components of the compound action potential (Erlanger and Gasser, 1937; Hursh, 1939; Rushton, 1951). Erlanger and Gasser (1924) showed that the compound action potential was made up of the summation of groups of action potentials. Each nerve fibre action potential travelled along the fibre at a different rate depending on the calibre and degree of myelination of that fibre. Thus simultaneously produced action potentials were displaced in time with increasing distance along the nerve. These could be recorded by a distant electrode as discrete peaks in the compound action potential. The first peak of the compound action potential therefore belongs to fibres conducting at the fastest rates. Since, as it has been shown in section 3:4:2, nerve regeneration is associated with significant changes in fibre diameter and myelination, these should

be reflected in significant changes in the compound action potential.

Figures 21-24 demonstrated that normal conduction velocities are never regained after any type of nerve repair and figure 25 showed that it was the fastest fibres that were lost after repair. This correlates with the loss of the largest diameter fibres reported previously. Conduction velocity increased with time, N-N sutured nerves reaching 58%, muscle grafted nerves 36% and cable grafted nerves 28% of normal velocity at 300 days. Cragg and Thomas (1964) showed that, even after a crush injury, conduction velocity only recovered to 75% of normal values after sixteen months. However, Berry, Grundfest and Hinsey (1944) found recovery to 80% of normal in cat nerves repaired by epineurial suture. It is difficult to compare results accurately between species and with older papers when the results presented here were measured with the most up-to-date recording equipment available. The percentage recoveries have as their denominator the fastest normal conduction velocity measured. The fastest fibres found in this study (70m/s) are faster than those reported in the rat by Birren and Wall (1956) and others (55-60m/s). This is probably due to greater accuracy in measuring the small distances involved on the oscilloscope screen. Slower fibres are easier to measure accurately because the distances involved are greater. This may explain why the percentage recovery is higher in the older papers quoted above. Gattuso *et al*, measured conduction

velocities of 55-70m/sec in the rat sciatic nerve (Dr J. Gattuso, personal communication, 1988).

There is no doubt from the results in section 3:3 that recovery of conduction velocity is poorer after a grafting procedure than after direct nerve suture. The muscle graft faired slightly better than the cable graft in terms of percentage recovery but the figures did not reach statistical significance.

Chronaxie is the time a voltage of twice rheobasic voltage has to be applied to a nerve to produce an action potential. It is a measure of the excitability of a nerve. It is useful because it can be standardised for comparison between groups. Rheobase and chronaxie can be read directly from the strength-duration curve. However the value of rheobase, as well as being a function of the true threshold of a nerve fibre population, is also a function of the preparation and so varies from rat to rat. It is therefore necessary to standardise the strength-duration curve to be able to compare values between rats. This was done by expressing the stimulus strength as volts/rheobase using a method derived by Glasby, Gattuso and Huang (1988).

Figure 32 shows that at times after 100 days there were no significant differences in the chronaxies of any of the experimental groups, or any of these groups compared to normal. This compares well with the work of Glasby, Gattuso and Huang (1988) who found no significant difference in chronaxie at 300 days between normal and after repair of rat sciatic nerve with muscle graft.

The chronaxie values presented here relate to that part of the sciatic nerve which had been repaired and so included that part of the fibre which had regenerated through the muscle graft. The slightly reduced level of myelination reported in this area of the regenerated nerve, compared to the distal nerve, was not reflected in a reduced conduction velocity or an increased chronaxie. It seems reasonable to conclude that this delay of myelination in the muscle graft is not significant in terms of nerve function.

In normal nerves the largest diameter fibres have the shortest chronaxie and are therefore easier to stimulate (Erlanger and Gasser, 1924). In regenerated nerve, fibre diameters were significantly smaller, and should have been reflected in a prolonged chronaxie time but, as has been shown, chronaxie was not significantly altered. In regenerated nerve smaller fibres have somehow become more excitable than normal fibres of that size. This may explain in part, the abnormal and often unpleasant sensations experienced after nerve repair, often in response to little stimulation. This is likely to be due, at least in part, to changes in the cell membrane, in particular at the node of Ranvier.

3:4:4 Muscle Function After Nerve Repair

Muscle function can be measured as an assessment of end organ function but some aspects of muscle physiology can also be used to give an insight into the degree of disorganisation of the nerve after regeneration. In

particular, twitch times can give a useful indication of mismatch of axons and end organs.

Muscle fibres can be divided into two broad categories, fast and slow twitch. EDL in the rat was found to consist of 90% fast twitch fibres (approximately 40 fast motor units) and was supplied by the peroneal nerve (Close, 1967). Soleus and gastrocnemius are predominantly slow muscles although gastrocnemius also has some fast fibres. Both are supplied by the tibial nerve. Fast twitch fibres were shown to have faster isometric twitch characteristics than slow twitch fibres (hence their names) and more recently this has been correlated with differences in enzyme content (Close, 1972). The experiments of Buller, Eccles and Eccles (1960a,b), among others, have shown that fast muscle fibres are innervated by fast motorneurones and slow twitch muscle fibres by slow motorneurones. Fast motorneurones had shorter after-hyperpolarisation periods and faster firing rates than slow motorneurones. Also, slow motorneurones were associated with smaller diameter axons (Eccles, Eccles and Lundberg, 1958). It was shown that by stimulating a denervated slow muscle to contract at high frequencies (mimicking fast motorneurone activity), the fibre content of that muscle could be changed and the muscle would display faster muscle fibre types. Denervated soleus muscle stimulated at low frequencies remained slow (Lomo, Westguard and Dahl, 1974). These changes were reflected in changes in twitch times. Cross innervation experiments have confirmed that

these findings also occur after nerve repair (Close, 1965; Barany and Close, 1971; Dum, O'Donovan and Toop *et al*, 1985a,b).

In this study repairs were made in the main sciatic trunk, containing both tibial and peroneal fascicles. Mismatch of tibial (predominantly slow motoneurones, destined for soleus and gastrocnemius) and peroneal fibres (fast and slow twitch but fibres destined for EDL predominantly fast) might occur in the graft or at the suture lines. This would be reflected in differences in muscle twitch times, since it has been shown that mammalian motor fibres show no selectivity during regeneration, according to muscle fibre type (Bernstein and Guth, 1961; Miledi and Stefani, 1969) or to individual muscles (Weiss and Hoag, 1946). This lack of selectivity has also been shown in goldfish extraocular muscles (Scherer, 1986). If appropriate fast motorneurones reinnervate EDL then twitch times will remain fast. As more inappropriate slow fibres reinnervate EDL, twitch times will increase.

Sensory fibres, although they were able to make contact with muscle **never** made functional contacts and were unable to elicit muscle contraction or to prevent muscle atrophy (Gutmann, 1945; Zalewski, 1970). Therefore if many sensory fibres inappropriately contact muscle this would be reflected in loss of muscle bulk. The degree of muscle sense-organ reinnervation has been explored further in chapters four and five.

Figure 35 showed that the duration of twitch was

significantly increased compared to normal after all methods of repair, up to and including 150 days. By 300 days however the N-N suture group had returned to normal. Grafted nerves never regained normal twitch times. There was no significant difference between muscle and cable grafted groups. Similar patterns applied in figures 36 and 37. Therefore it can be concluded that after nerve repair by muscle or cable graft, EDL muscle had become significantly slower, reflecting inappropriate slow motorneurone innervation. It could be argued however that since slow motorneurones are associated with small diameter fibres and fibre size is reduced following nerve regeneration, that slowing of previously fast motorneurones is a consequence of this alone. Since after N-N suture fibre diameters are significantly reduced (figure 18), yet twitch times recover to normal, this seems unlikely. Also, Kuno, Miyata and Munoz-Martinez (1974) reported that soleus motorneurones could become faster after axotomy. It would therefore seem correct to assume that a decrease in twitch time does indeed reflect disorganisation of motor innervation. It would be interesting to look at the muscle fibres in regenerated muscle using histochemical methods to stain for fast and slow muscle fibre types.

The force generated by reinnervated muscle was looked at in several ways since no one method is ideal. The isometric tension generated by a muscle was said to vary approximately as the number of active motor units (Weiss and Hoag, 1946). This can be used as a measure of

regeneration if a supramaximal stimulus is used to excite all motor units at the same time. The maximum isometric twitch tension can easily be measured (figure 38) but does not take into account how long this tension can be sustained. The area under the isometric myogram curve (known as the time-tension integral) gives a measure of total tension for any given twitch (figure 39) giving a better estimate of the force generated (Hartree and Hill, 1921). Cardiac physiologists when measuring the work of the heart also use the time-tension integral (the area under the pressure trace) but often use the time-tension index which takes into account the heart rate (Burton, 1957). The equivalent measure in skeletal muscle would be the area under the isometric tension curve divided by the duration of the twitch (units = Newtons). This is displayed in figure 40. It gives an "average" tension which is easily compared from group to group. All three graphs have similar appearances. Time-tension index shows no significant difference between cable and muscle grafted muscle function after 300 days. Muscle function appears to be particularly poor at 50 days after repair in the muscle grafted group. This probably reflects axonal delay within the graft. By 100 days the muscle grafted group have caught up with the others.

Area under the integrated EMG is another way at looking at muscle power (figure 41). The RMS integral of a waveform (in this case the EMG) represents the power of the signal. It has been shown that the voltage-time

integral of the surface EMG recorded in an actively contracting human muscle varied as the force of the isometric contraction (Lippold, 1952; Edwards and Lippold, 1956) and Leuman and Ritchie (1983) stated that the integrated mean voltage during graded muscle contraction had a linear relation to the isometric tension. Loofbourrow (1948) used the integrated EMG as a relative measure of the output of contracting muscle.

The time-tension index was the only method which brought out the poor performance of the muscle grafted group at 50 days. This correlated well with the degree of muscle atrophy seen at 50 days. Again the muscle grafted group had lost significantly more bulk than the others, although this was quickly regained so that no difference could be found between muscle and cable grafted groups at 100 days and over.

The conduction across muscle records the velocity of the muscle action potential as it travels a certain distance through the muscle membranes (surface membrane, t-tubules and sarcoplasmic reticulum) to a recording electrode. A small part of this will also reflect conduction in the most distal parts of nerve fibres within the muscle. This velocity was never significantly different from normal even in the most atrophied muscles. However the amplitude of the muscle action potential was reduced in severely atrophied muscle and, as we have seen, did not lead to a normal level of contraction. The mechanism of this is unclear but probably works either by decreased calcium release within the muscle or, more likely, a

reduction in the amount of myofibrillar protein within the muscle fibre.

In summary, there were no significant differences to be found at 300 days in the level of nerve or muscle function or nerve morphology after muscle graft or cable graft, although neither had returned to normal by this time.

CHAPTER FOUR

THE SENSORY REINNERVATION OF MUSCLE

4:1 The motor reinnervation of EDL muscle after sciatic nerve repair has been looked at in some detail in chapter three. The importance of muscle sensory reinnervation must not be overlooked. Motor function is intimately associated with control and coordination. Any degree of motor power is useless unless it can be controlled adequately. Patients complained more often about loss of fine control and coordination (especially in the hand) and sensory disturbance than about loss of power after nerve repair (MRC Special Report No. 282, 1954).

Several groups of sensory nerve fibres can be found within skeletal muscle. They are summarised in Table 4:1. These fibres have their cell bodies within dorsal root ganglia.

Table 4:1 Classification of Muscle Afferent Fibres

Type of Sheath	Myelinated			Non-myelinated
Fibre Diameter(μm)	22		1.5	2 – 0.1
Conduction Velocity (m/s)	100	60	30 4	0.5
Lloyd Classification *	I	II	III	IV
Muscle Afferents	a. Primary Spindle Ending b. Golgi Tendon Organ	Secondary Spindle Ending	Free Ending (nocice-ptor) Paciniform Endings (pressure)	Free Ending (nocic-eptor, thermal, etc.)
Erlanger and Gasser Classification **	Aα		Aδ	C

* Lloyd (1948)

** Erlanger and Gasser (1937)

When peripheral nerve is severed, nerve cell bodies undergo a series of reactive changes known as chromatolysis (this process has already been mentioned in chapter one). Descriptions of these changes can be found in any standard textbook (Sunderland, 1978). The main light microscope changes described include:

1) loss of Nissl substance which led to a marked loss of cell basophilia (Ranson, 1909; Engh and Schofield, 1972)
2) swelling of the cell body which started at 3-4 days after injury and became maximal between 10 and 20 days (Ducker, 1972)
3) migration of the nucleus to the periphery of the cell (Ranson, 1909; Schmalbruch, 1987b).

Changes in function can also be observed:

4) increased enzyme activity leading to increased RNA and protein synthesis (Ducker, 1972).

These changes were seen to affect motorneurones as well as sensory neurones (Eccles, Libet and Young, 1958; Bowe, Yu and Waxman, 1988) although sensory neurones appeared to be affected more severely (Hoffer, Stein and Gordon, 1979). Chromatolysis persisted as long as there was active axon regeneration and seemed to represent a non-specific manifestation of cell insult (Ducker, 1972). The return towards normal cell morphology was characteristic of the recovery and maturation phase of peripheral nerve regeneration and occurred only if axon growth was successful (Cavanaugh, 1951). However, not all cells regained normal cell morphology after regeneration. Some remained small and

atrophied while others appeared to have died (Ranson, 1909; Risling, Aldskogius, Hildebrand *et al*, 1983; Janig and McLachlan, 1984; Ygge and Aldskogius, 1984; Ardvidsonn, Ygge and Grant, 1986; Schmalbruch, 1987b; Peyronnard, Charron, Lavoie *et al*, 1986b,1988; Ygge, 1989). The degree of cell loss produced by peripheral neurotomy in various species has been reported as being in the order of 11-53% (see table 4:2 for details).

Table 4:2 Degree of Cell Loss After Neurotomy

% loss	Author	Year	Nerve studied
52%	Ranson	1909	Rat C2 nerve
11-53%	Risling	1983	Adult cat sciatic
51%	Janig *et al*	1984	Adult cat peroneal
15-30%	Ardvidson *et al*	1986	Adult rat sciatic
7%	Ygge *et al*	1989	Distal rat sciatic
27%	Ygge *et al*	1989	Proximal rat sciatic

The precise factors which determine whether a cell will survive, atrophy or die remain uncertain. Factors which are known to influence the process include:

1) The Level of Injury

Lesions nearer to the cell body produce more cell loss than lesions further away from the cell body (Carlson, Lais and Dyck, 1979; Ygge, 1989).

2) Age

Neonatal rats experienced greater cell loss than older

rats for a given nerve lesion (Schmalbruch, 1987b).

3) Severity of the Lesion

Crush injuries produce less cell loss than neurotomy (Peyronnard, Charron, Lavoie *et al*, 1988).

4) Locally Supplied Growth Factors

Sciatic nerve section was shown to reduce substance P in DRG cells and the dorsal horn of the spinal cord (Tessler, Himes, Krieger *et al*, 1985). Also the presence of exogenously supplied NGF prevented DRG cell death in response to sciatic nerve section in the rat (Rich, Luszczynski, Osborne *et al*, 1987).

Other factors seemed to produce more variable effects:

5) Repair of the Lesion

Ranson (1906) stated that cells were lost whether or not regeneration took place. However Schmalbruch (1987b) noted that in rats where regeneration of muscle nerve supply was almost perfect, there was little if any DRG cell loss and Carlson, Lais and Dyck (1979) found that in cats, cell loss was greater in permanently transected nerves than in repaired nerves.

The <u>method</u> of repair also seems to influence the degree of cell loss. A single neurotomy repaired by epineurial suture (in cat common peroneal nerve) resulted in a reduction of muscle spindle and tendon organ afferents to 24% and 45% of normal respectively. This was compared to double neurotomy and repair (full thickness autologous nerve graft) which resulted in the loss of 90% of both types of afferents (Banks, Barker and Brown, 1985). Barker, Berry and Scott, (1990) found

no detrimental effect on spindle sensory reinnervation after delayed versus immediate epineurial suture of cat sciatic nerve. However Peyronnard, Charron, Lavoie *et al*, (1986b) found that, in the rat, DRG cell counts were much reduced after four weeks, if repair of the sciatic nerve was not undertaken before this time.

It is known that cutaneous and muscle afferents have a precise somatotopic organisation within the dorsal horn of the spinal cord (Brushart, Henry and Mesulam, 1981; Devor and Wall 1978, 1981) and that this organisation is destroyed after nerve section (Devor and Wall, 1981) and even after precise fascicular repair (Brushart, Henry and Mesulam, 1981). It is less certain whether these afferents also have a somatotopic orgaisation within the DRG. It is well recognised that there is a degree of somatotopic organisation within the trigeminal ganglion (Mazza and Dixon, 1972; Darian-Smith, 1973; Furstman, Saporta and Kruger, 1975) and within the vagal nuclei (Molhant, 1913; Lieberman, 1968). However the presence of somatotopic organisation within the DRG itself has not been investigated fully. Burton and McFarlane (1973) investigated the distribution of <u>cutaneous</u> afferents within the L7 DRG of the cat using injections of ^{3}H proline and electrophysiological recording of DRG cells. They found that there was a shifting pattern of cutaneous receptive fields within the DRG. Preaxial foot and leg fields were represented laterally within the ganglion and postaxial foot and leg fields were represented in the medial half of the ganglion. Ygge

(1984) found no evidence of precise anatomical somatotopic organisation within the DRG of the thoracic nerves of the rat. However he did not dismiss the idea that there might be some degree of organisation according to sensory modality. The presence of a somatotopic arrangement of muscle afferents within the DRG is still a matter of some debate. Peyronnard, Charron, Lavoie *et al*, (1986a) remarked on a degree of clustering of muscle afferents within the DRG of normal rats, shown by HRP, but did not go on to investigate this further.

It can be seen from the above account that many aspects of sensory nerve regeneration require clarification. Much of the work has been done on varying species at different ages and the nature of the nerve lesion has not always been clear. The experiments detailed in this chapter were designed to answer the following questions.

1) What degree of muscle sensory cell loss and atrophy is associated with various methods of nerve repair?
2) What effect does nerve repair have on the muscle afferent organisation of the DRG, at a spinal level and within the DRG itself (*i.e.* does somatotopic organisation of muscle afferents exist)?
3) Do muscle grafted nerves differ in these respects from nerves repaired by conventional means?

4:2 Materials and Methods

(See Appendix 7 for details of solutions)

Twenty-six rats were studied. A 1cm gap in the left sciatic nerve was repaired in one of three ways.

1) Direct epineurial suture (5 rats)
2) Autologous freeze-thawed muscle graft (5 rats)
3) Three-strand cable graft (5 rats)

The techniques have been described in Chapter 2. The rats were allowed to recover for 300 days.

Five unoperated rats were used as normals for comparison.

4:2:1 Control Procedures

Six rats were used as controls for HRP leakage. In three of these control rats HRP solution was injected into the left EDL muscle as described below. The muscle was then totally denervated by cutting all the muscle branches to it from the peroneal nerve. The tibial and sural nerves were cut and ligated with a silk suture. No reaction product was found in the DRG of L2-L6 after 48 hours in two rats. However in one rat one stained cell was found in L2 DRG. This was probably due to HRP leakage into the overlying tibialis anterior muscle. This rat had chewed through its skin sutures and its wound was open which may explain the leakage.

In the remaining three rats HRP solution was injected into the EDL muscle and the peroneal nerve was ligated at the knee with 3/0 silk. The tibial and sural nerves were cut. No reaction product was found in dorsal root

ganglia L2-L6 after 48 hours in all three rats. This was taken to show that there was no significant leakage of HRP into surrounding muscles and that denervation of the tibial and sural territories was complete.

4:2:2 Experimental Procedures

At 300 days the rats were anaesthetised as described previously. The EDL muscle on the operated side was exposed by making a skin incision on the lateral aspect of the lower limb from the knee, running onto the dorsum of the foot thus exposing the tibialis anterior muscle and its tendon. The tendon was cut and pulled proximally to expose the tendons of EDL running under the extensor retinaculum. These were followed upwards to the muscle belly, being careful not to disturb the neurovascular bundles entering the muscle. The muscle belly was injected with 50µl of 20% HRP solution using a Hamilton microlitre syringe, making sure that all areas of the muscle became stained with solution. HRP is taken up at neuromuscular junctions and sensory nerve terminals and transported retrogradely to the cell body (Honchien, Kao and Tan, 1980; Mesulam, 1982). The injected solution was spread further within the muscle when the rat awoke and started to use the limb. Care was taken to avoid spillage and leakage into other muscles. Tibialis anterior muscle was denervated to make absolutely sure that any leakage onto it would not be transported into the DRG. The tibial and sural nerves were divided as they left the main sciatic trunk. A

piece of nerve was removed to make sure there was no regeneration or leakage across the gap. The wound was closed with 6\0 Vicryl. The rats recovered and were allowed free access to food and water. Between 40 and 48 hours after this procedure the rats were again anaesthetised and killed by perfusing them through the heart in the following way:

1) The chest was opened. 0.1% sodium nitrite was injected into the left ventricle to produce vasodilatation.

2) 250 ml of normal saline was infused to flush out red blood cells.

3) The left ventricle was then perfused with 500 mls of 1.25% glutaraldehyde, 1% paraformaldehyde fixative at 4°C, followed by 100 ml of sucrose buffer solution at 4°C.

The dorsal root ganglia of L2-L6 were removed by performing a thoracolumbar laminectomy, under a dissecting microscope. T13 spinal nerve was identified by finding it running under the last rib. T13 dorsal root could be followed backwards to the spinal cord. The lumbar dorsal roots could be identified by counting downwards from T13. The DRG could be seen as fusiform swellings of the dorsal roots, lying in the spinal foramina. They were removed, labelled and stored individually in sucrose buffer at 4°C. The ganglia were

frozen in embedding medium, orientated longitudinally and cut serially at 40μm in a Reichart-Jung freezing microtome. Each section was floated out into phosphate buffered saline (PBS) solution and processed as follows.

4:2:3 Tetramethylbenzidine Method of HRP Processing

1) Free-floating sections were rinsed in distilled water (4x15 seconds).

2) They were immersed in tetramethylbenzidine (TMB) incubation solution for 20 minutes at room temperature.

3) Enzymatic reaction: 5ml of freshly prepared 0.3% hydrogen peroxide solution was added to each 200 ml of pre-reaction soak and the sections incubated in this solution for 20 minutes or until the solution turned green. (Blue-green granules appeared in the solution at that time.)

4) Sections were rinsed in distilled water and stored in PBS solution until they could be mounted on chrome alum coated slides and air dried.

5) The slides were counterstained with 1% buffered neutral red solution for two minutes and rinsed in distilled water until the water ran clear. They were dehydrated in a graded series of alcohols (10 seconds each, 20 seconds in absolute) and cleared in xylene (twice x 2 minutes). They were mounted in DPX and stored in the

fridge at 7°C. At this temperature the reaction granules will remain for about two months. At higher temperatures they will fade more rapidly.

4:2:4 Cell Counts

Each section was looked at and only stained cells which showed a nucleolar profile were counted. Since the nucleolus is single and central (Landon, 1976) this ensured that no cell was counted twice in successive sections unless the nucleolus had been split by the microtome knife. A correction factor for split nucleoli was applied, taking into account the size of the nucleoli and the thickness of the section (Konigsmark, 1970). The formula for the correction factor was

$$CF = t/(t+2r)$$

where t = section thickness in microns and

r = mean radius of the unit counted.

In this case t = 40 and r = 2 (radius of the nucleolus), giving a correction factor of 0.91. Absolute counts were multiplied by this factor to give actual counts.

Cell diameters were measured using the Magiscan, a computerised morphometric analysis system (Joyce-Loebl). The data were handled using Lotus "Symphony" Software on a Tandon personal computer system as described in Chapter 2.

Each section was looked at under a light microscope (Leitz, Germany) with a drawing attachment. Scale drawings were made of serial sections and the positions of stained cells were accurately marked. Each section

was copied onto transparent acetate sheets which could then be superimposed to give a three-dimensional image of each DRG. In this way the distribution of stained cells within a given ganglion could be seen.

4:3 Results

All rats made a good recovery by 300 days although function never quite returned to normal. The toe-spreading reflex could be elicited in all animals and was taken to reflect good sciatic nerve regeneration. One cable-grafted rat died 24 hours after injection of HRP. No cause could be found for this.

Figure 52 shows the average number of labelled cells in each DRG, from L2-L6, after various methods of repair. No labelled cells were ever found in L2 ganglion. In the normal rat labelled cells were found predominantly at two levels, L4 and L5, with few cells outwith this range. In any one rat labelled cells were found at only three levels, L3-L5 or L4-L6. After direct nerve suture this pattern could still be seen but with fewer cells in L4 and L5 and slightly more in L3 and L6. After cable grafting or muscle grafting the spread of cells was much greater with significant numbers found outwith L4 and L5. In any individual rat labelled cells could be found at four levels. This is presumably due to misdirection of fibres within the graft leading to disorganisation of sensory reinnervation.

Figure 53 shows the mean <u>total</u> numbers of labelled cells over levels L2-L6 in normal rats and after repair by

direct epineurial suture, cable and muscle grafts. After repair by N-N suture the mean cell loss was 29% (range 24-36%) compared to normal. After both cable and muscle graft the mean cell loss was 36% (muscle graft range 32-42%, cable graft range 26-45%). Cell counts after all three methods of repair were significantly different from normal ($p<0.0005$). However, cell counts after all types of repair were not significantly different from each other (Table 4:3) although absolute counts after N-N suture were higher than after grafting procedures.

Figure 54 shows the frequency distributions of DRG cell diameters in normal rats and after sciatic nerve repair by N-N suture, cable or muscle grafts. Repair by any of these methods was followed by a reduction in mean cell size. Large cells (diameter > 60μm) were seen less often after nerve repair and were replaced by smaller cells. After all three types of repair cell diameter was significantly different from normal. There was no significant difference between muscle and cable grafted rats but both of these groups were significantly different from rats which had undergone repair by N-N suture (Table 4:4).

Figure 55 is a photograph of a normal large L4 DRG cell containing HRP reaction product, visible as dark staining granules in the cytoplasm. The paler nuclear shadow contains a darkly staining central nucleolus.

Figure 56 shows one of the few large cells found 300 days after nerve repair with freeze-thawed, co-axial,

autologous muscle graft.

Figure 57 shows a smaller stained cell in L5 DRG 300 days after repair by muscle graft. A larger cell can be seen (out of focus) deeper in the section, to the left.

Figure 62 shows the arrangement of DRG cells within a typical L4 DRG. The cells lie in irregular cords within the ganglion, intermingled with axons. Figures 58,59,60 and 61 show the typical arrangement of labelled cells within the L4 DRG. In the normal rat (Figure 58) there is a definite clustering of afferents from EDL muscle towards the superior pole of the ganglion. This pattern is lost after nerve repair, reflecting disorganisation of afferent regeneration (Figures 59, 60, 61).

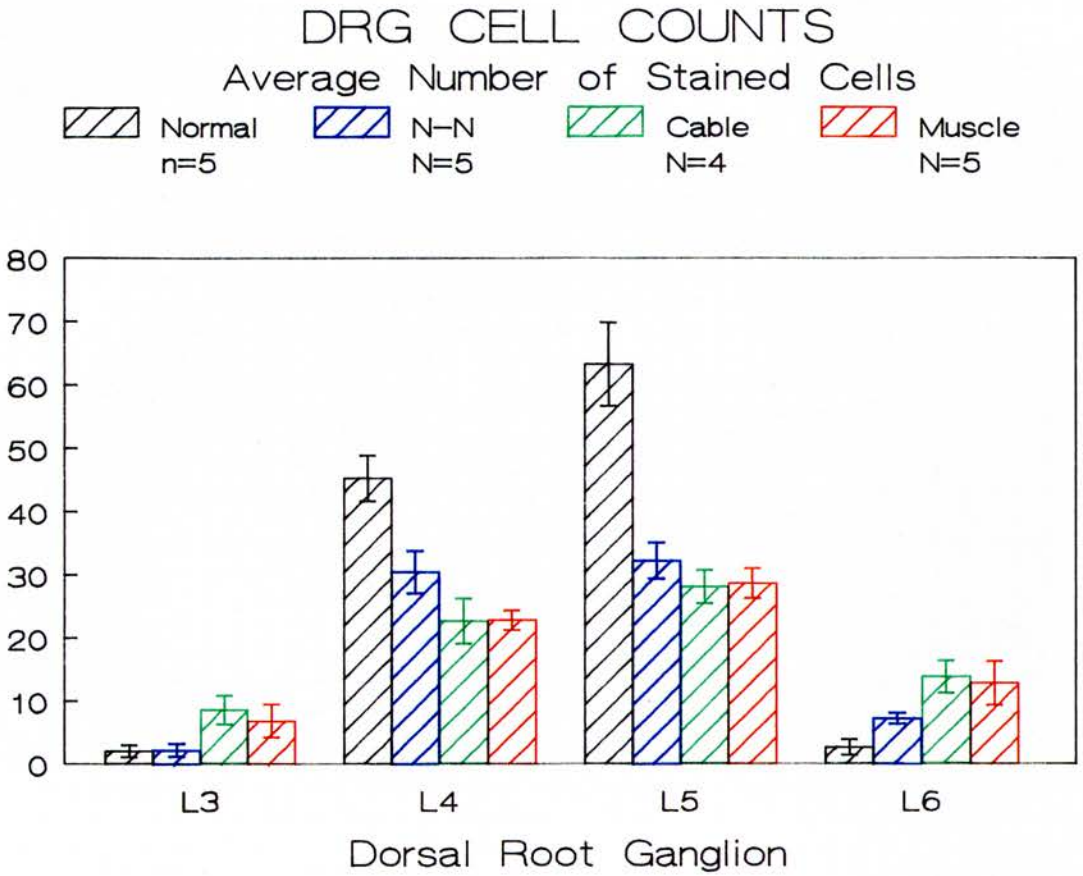


Figure 52: The average number of HRP-labelled cells in L2-L6 DRG after various methods of nerve repair. Note the more variable distribution across spinal segments after nerve repair.

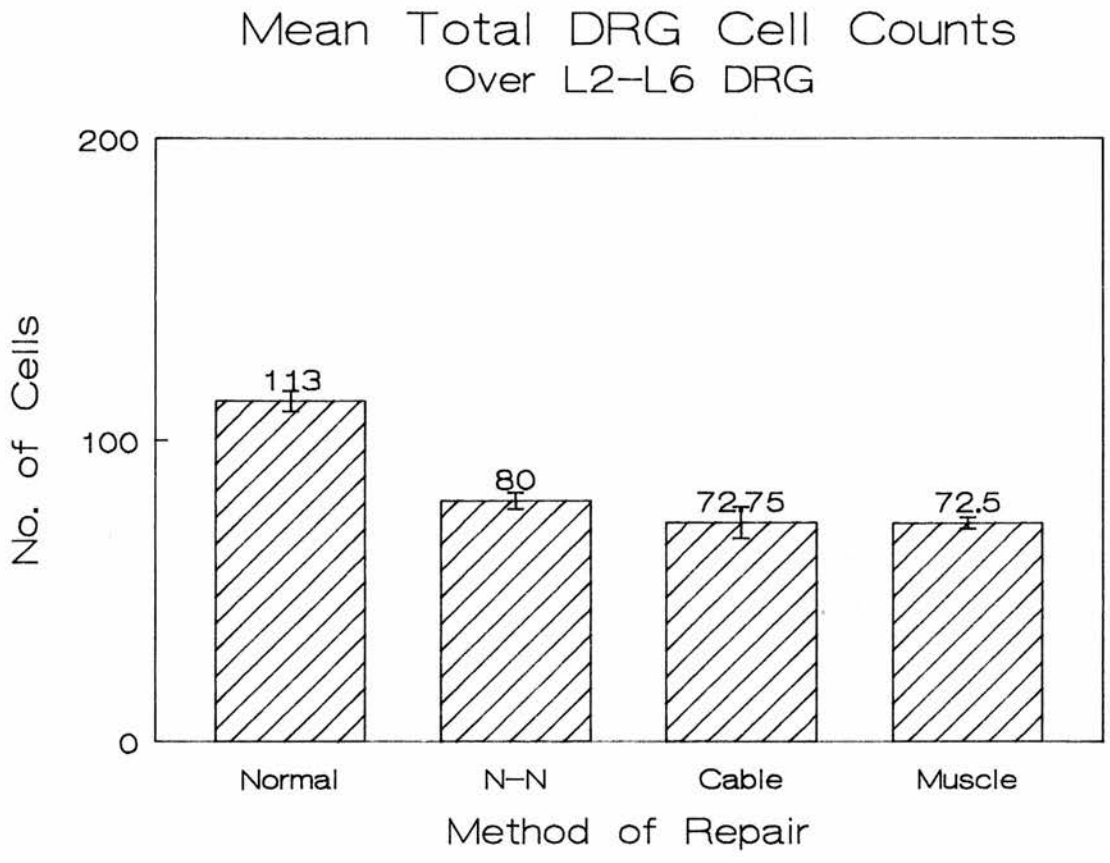


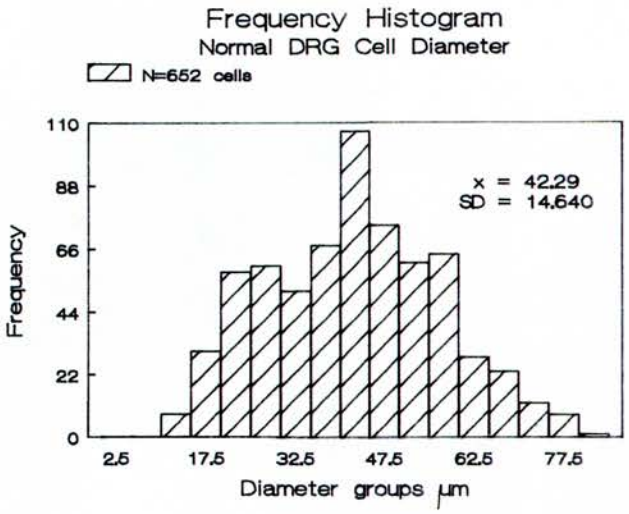
Figure 53: The mean total number of HRP-labelled cells in L2-L6 DRG after nerve repair. The average cell loss after N-N suture was 29%. The average loss after cable or muscle grafting was 36%.

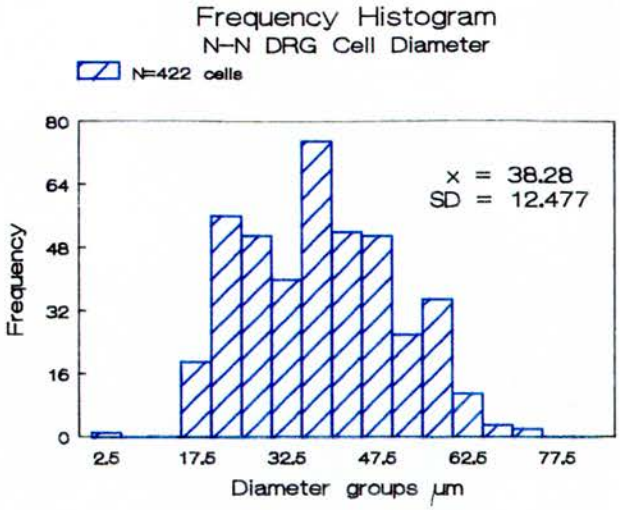
Table 4:3 Total DRG Cell Counts: P – Values

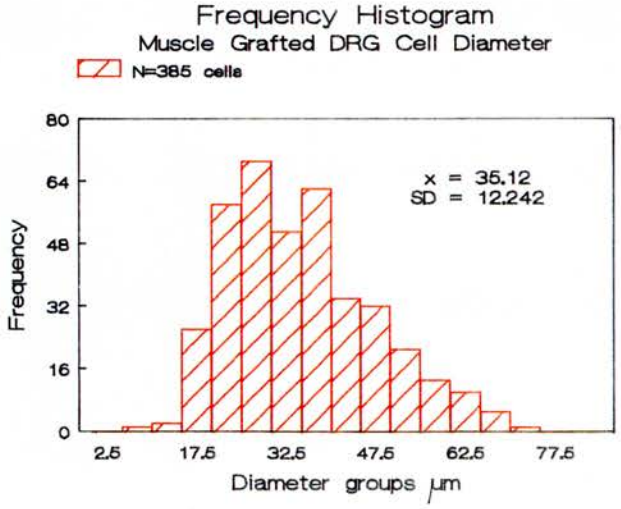
	Normal	N–N	Muscle	Cable
Normal	*	p<0.0005	p<0.0005	p<0.0005
N–N	p<0.0005	*	NS	NS
Muscle	p<0.0005	NS	*	NS
Cable	p<0.0005	NS	NS	*

Table 4:4 DRG Cell Diameter: P – Values

	Normal	N–N	Muscle	Cable
Normal	*	p < 0.01	p<0.0005	p<0.0005
N–N	p < 0.01	*	p < 0.01	p < 0.01
Muscle	p<0.0005	p < 0.01	*	NS
Cable	p<0.0005	p < 0.01	NS	*







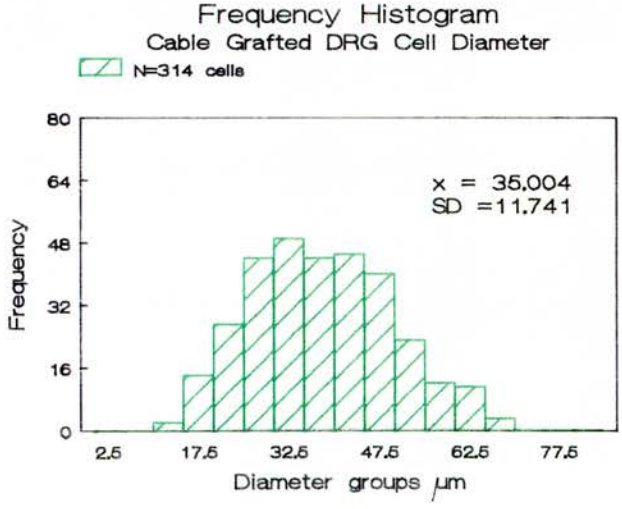


Figure 54: Frequency distribution of labelled DRG cell diameters in normal rats and 300 days after sciatic nerve repair by N-N suture, muscle or cable graft.

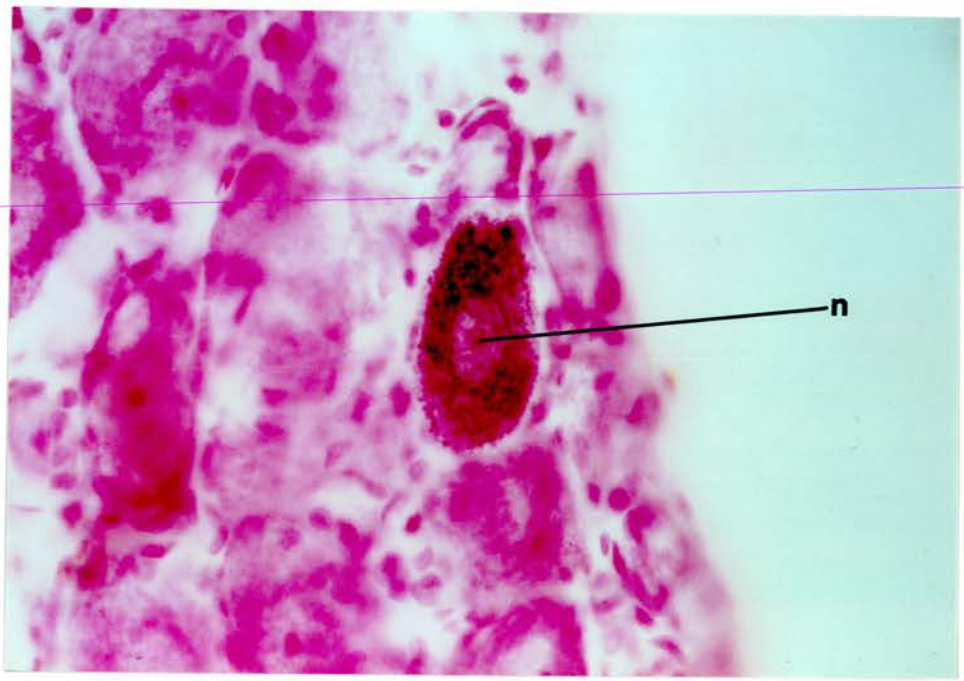


Figure 55: A 40µm frozen section of normal L4 DRG showing a large DRG cell containing HRP granules. Note the nucleolar profile (n) within the cell. (x 400)

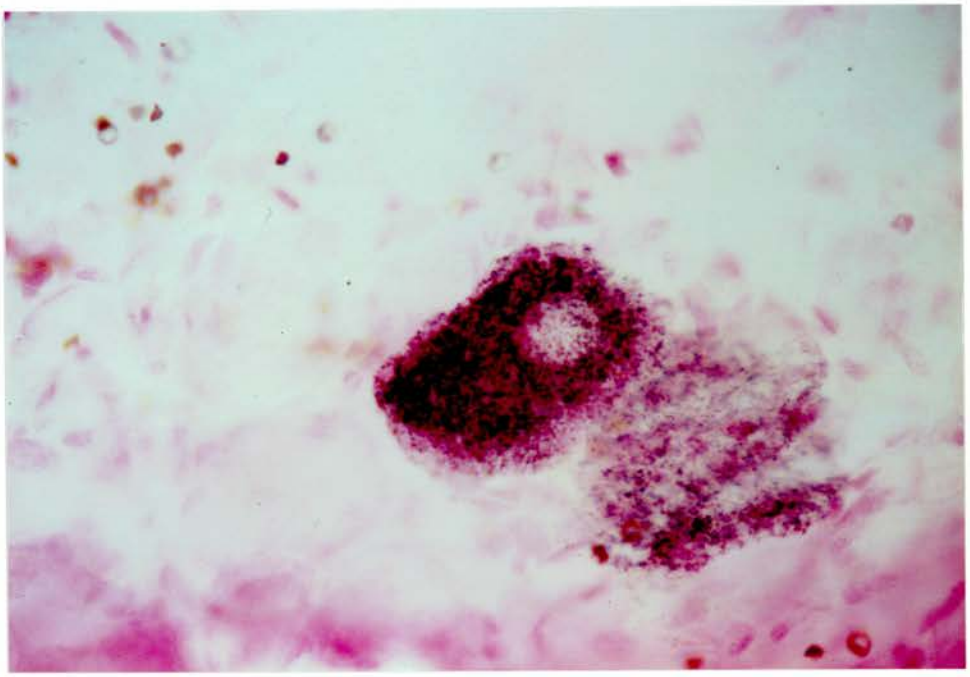


Figure 56: A 40μm frozen section of L4 DRG, 300 days after sciatic nerve repair with freeze-thawed muscle graft. This shows one of the few large cells found after nerve repair. (x600)

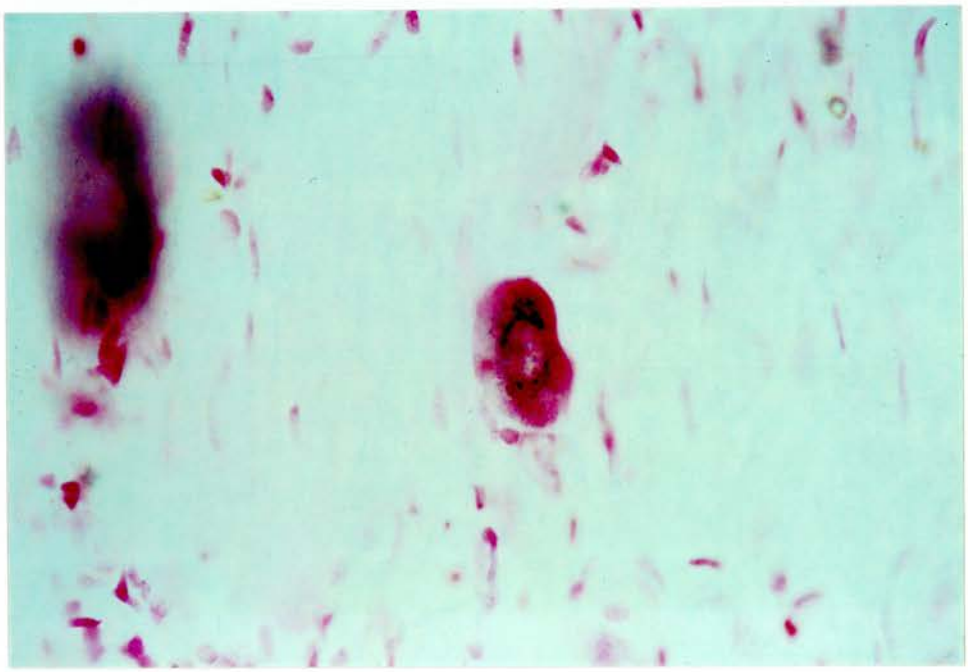


Figure 57: A 40μm frozen section of L5 DRG, 300 days after sciatic nerve repair with freeze-thawed muscle graft. This shows a more typical small-diameter cell. (x700)

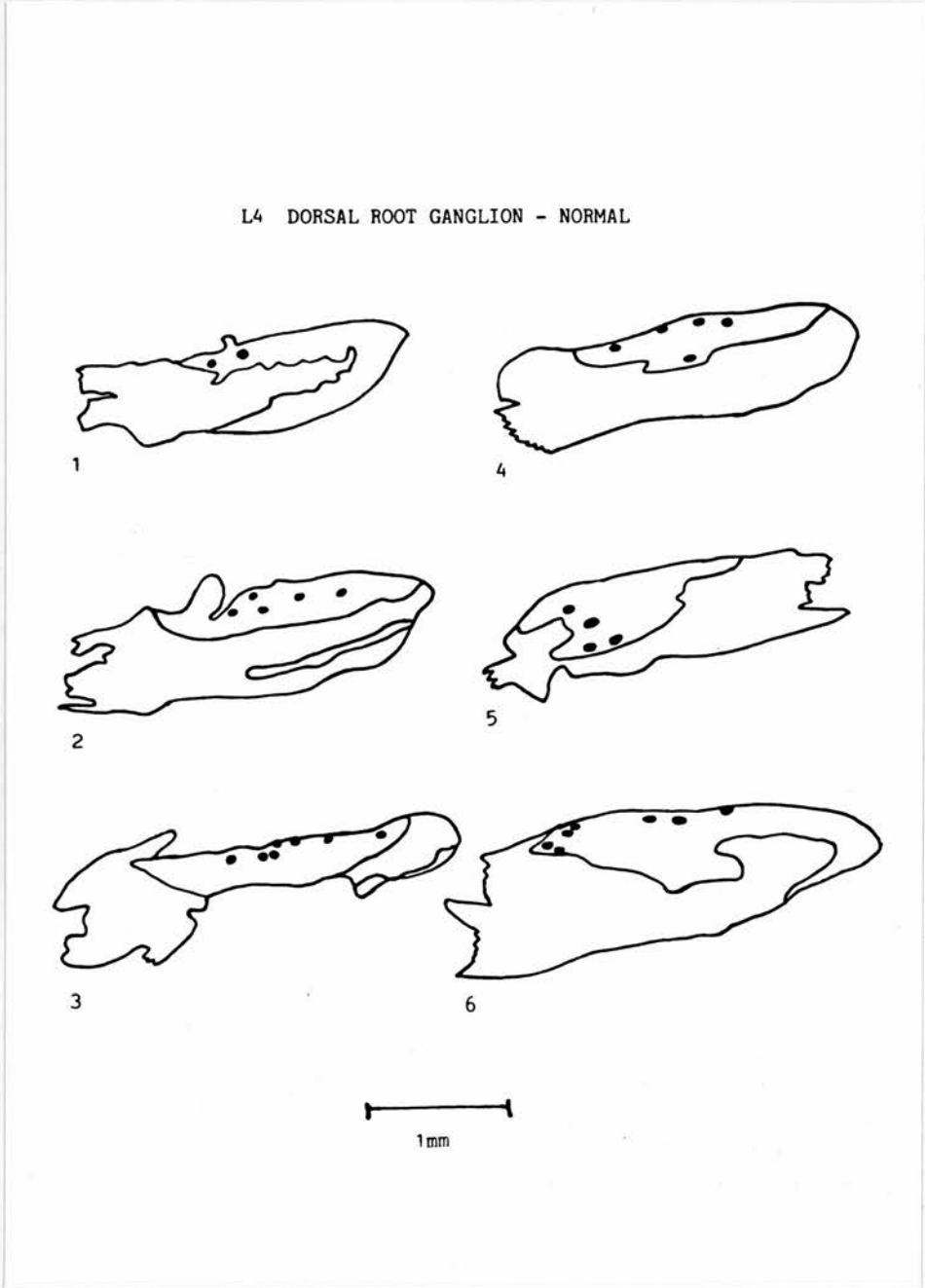


Figure 58: Diagrammatic representation of 40µm serial sections through a normal L4 DRG showing the distribution of HRP-labelled cells. Note the clustering of cells from EDL in the superior pole of the ganglion (continued overleaf).

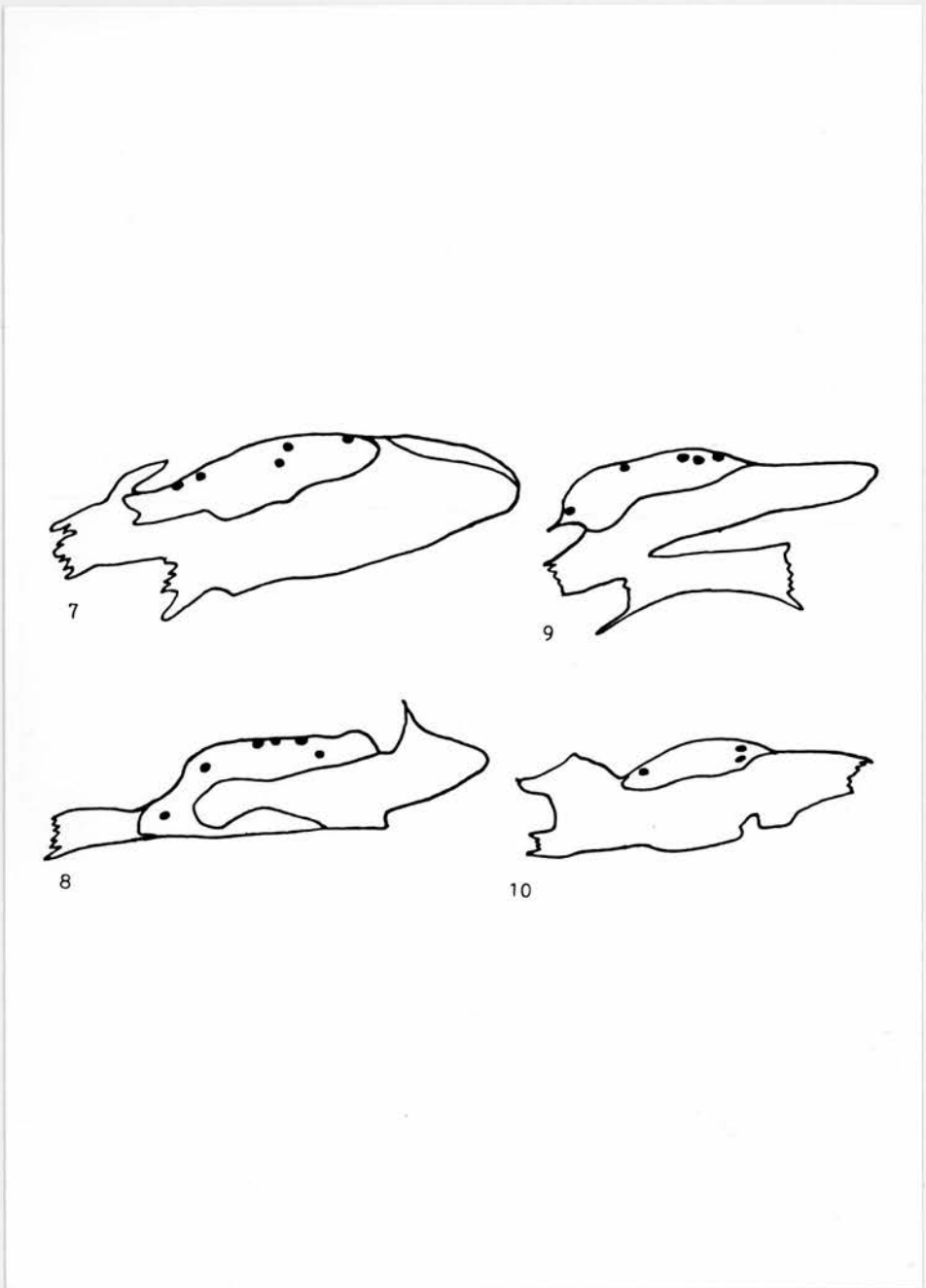


Figure 58: continued from previous page.

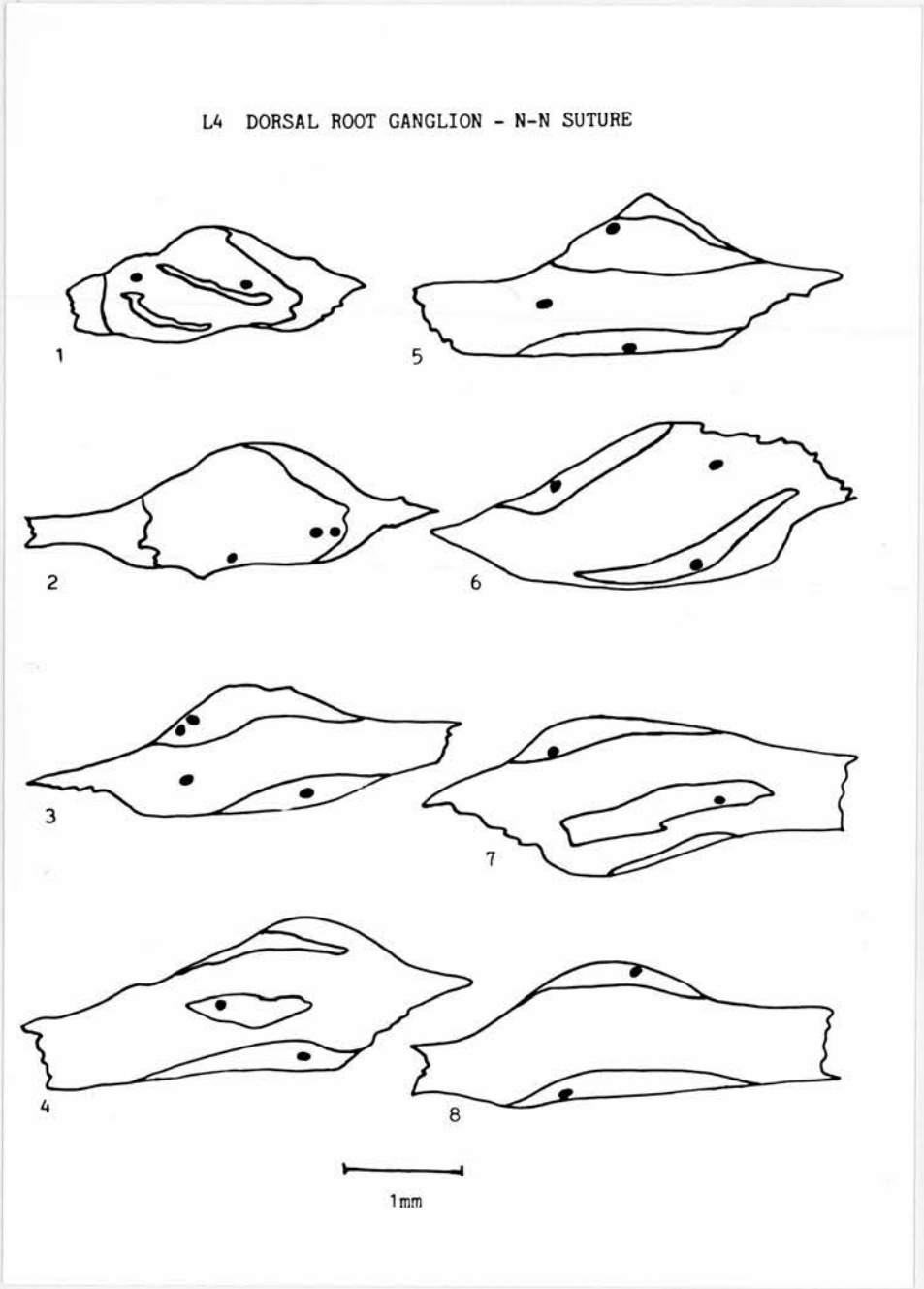


Figure 59: Diagramatic representation of 40μm serial sections through L4 DRG, 300 days after repair of the sciatic nerve by direct nerve suture. There is no clustering of labelled cells (continued overleaf).

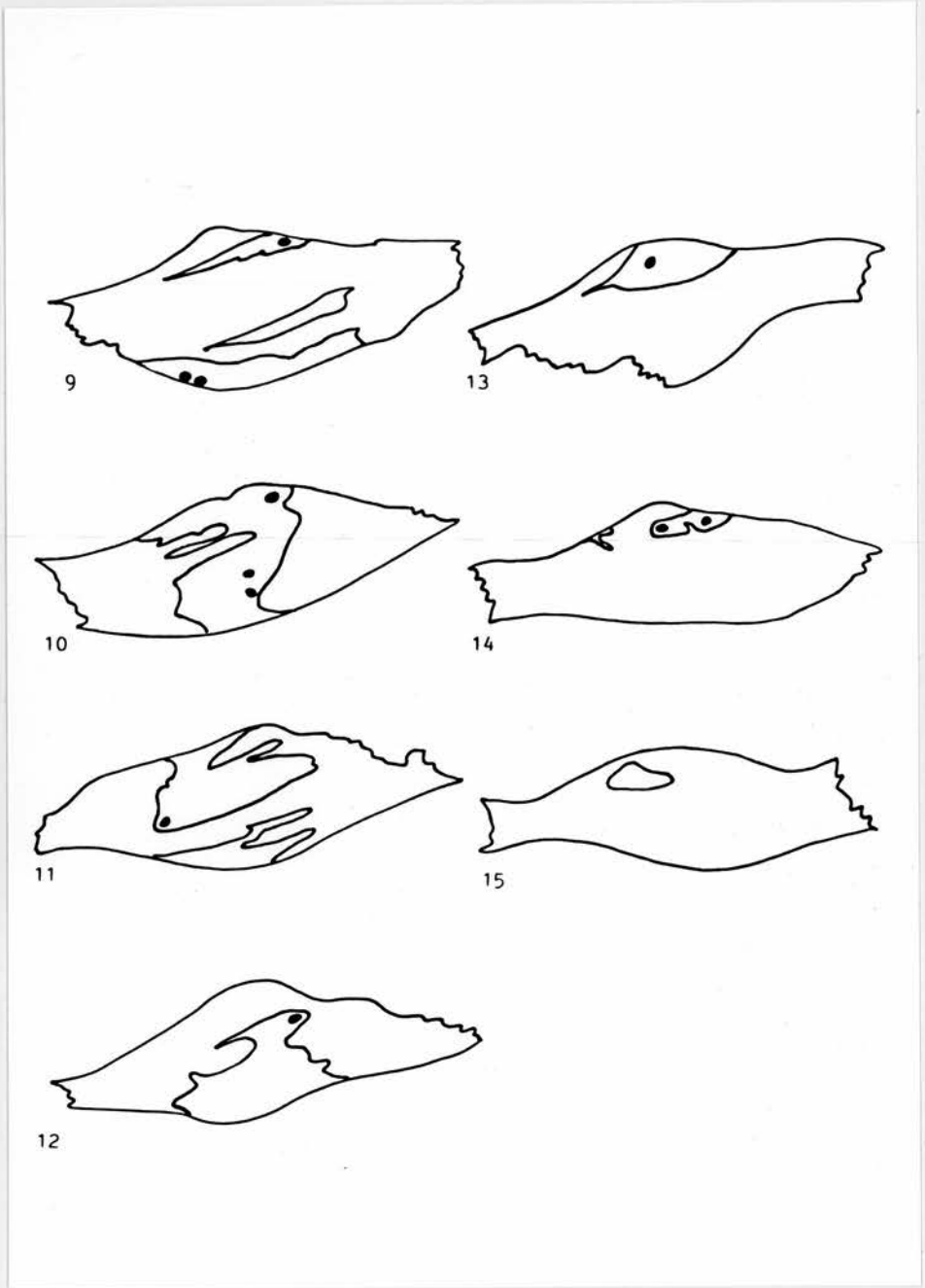


Figure 59: continued from previous page.

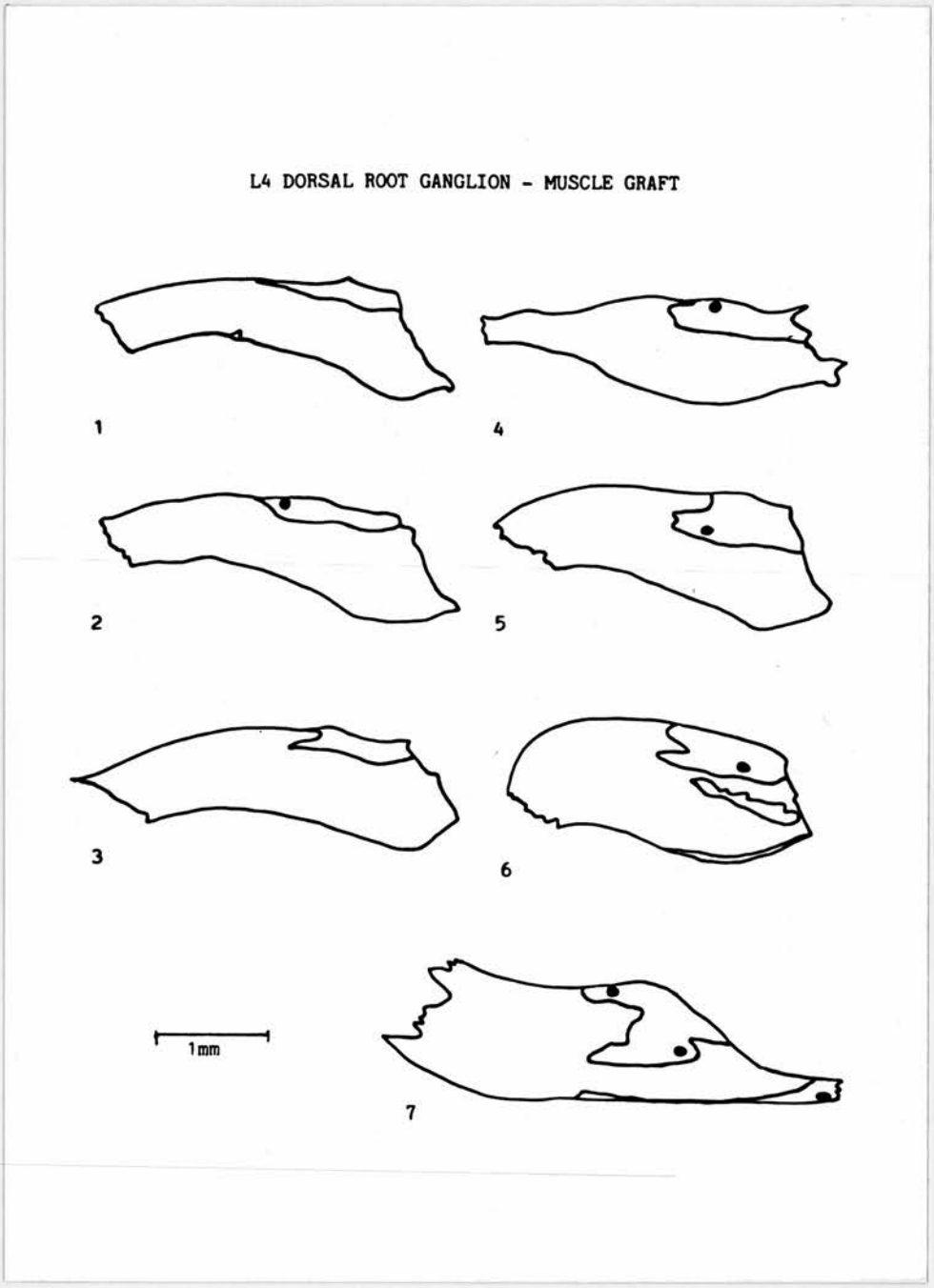
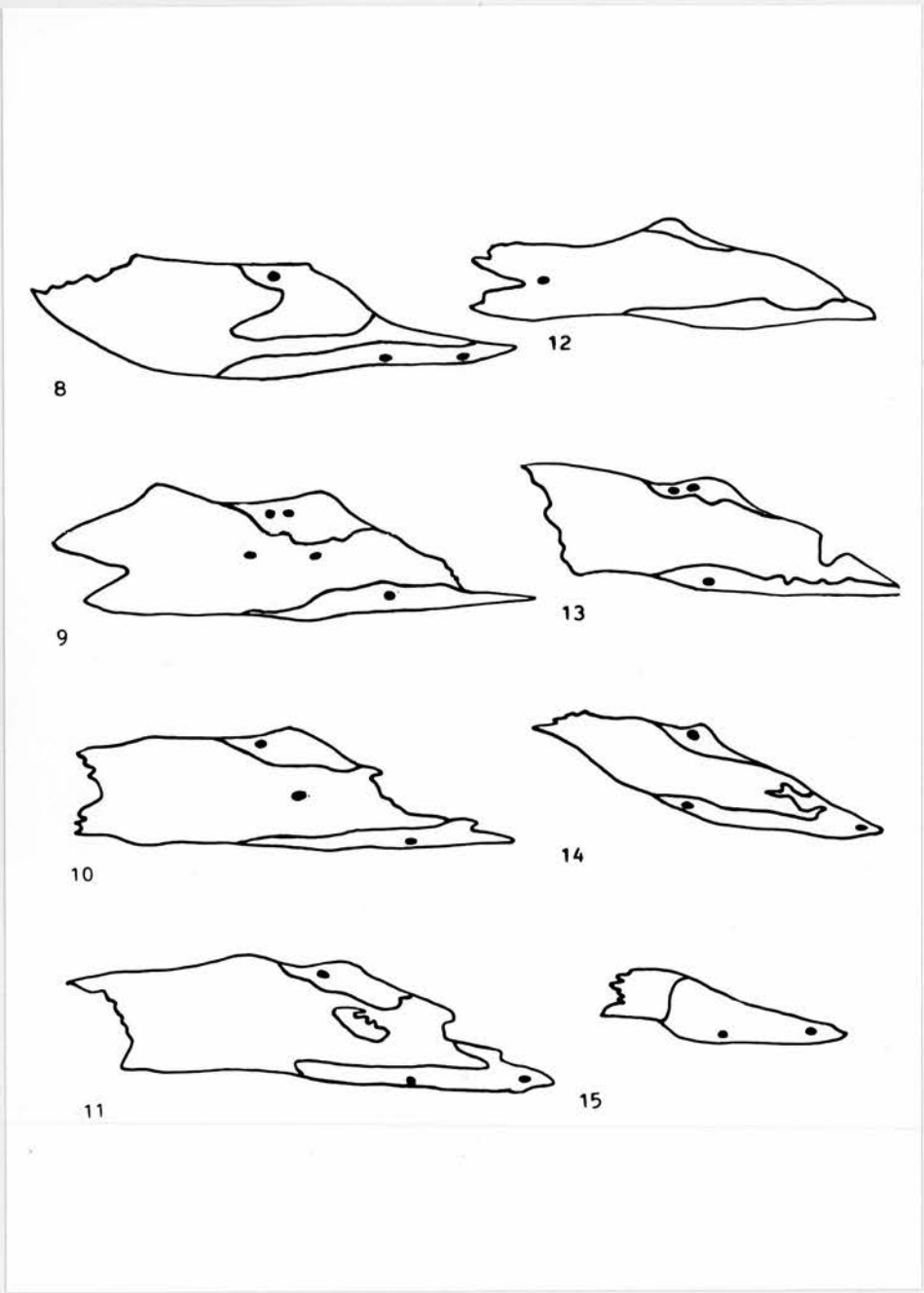


Figure 60: Diagrammatic representation of 40µm serial sections through L4 DRG, 300 days after repair of the sciatic nerve by freeze-thawed muscle graft (continued overleaf).



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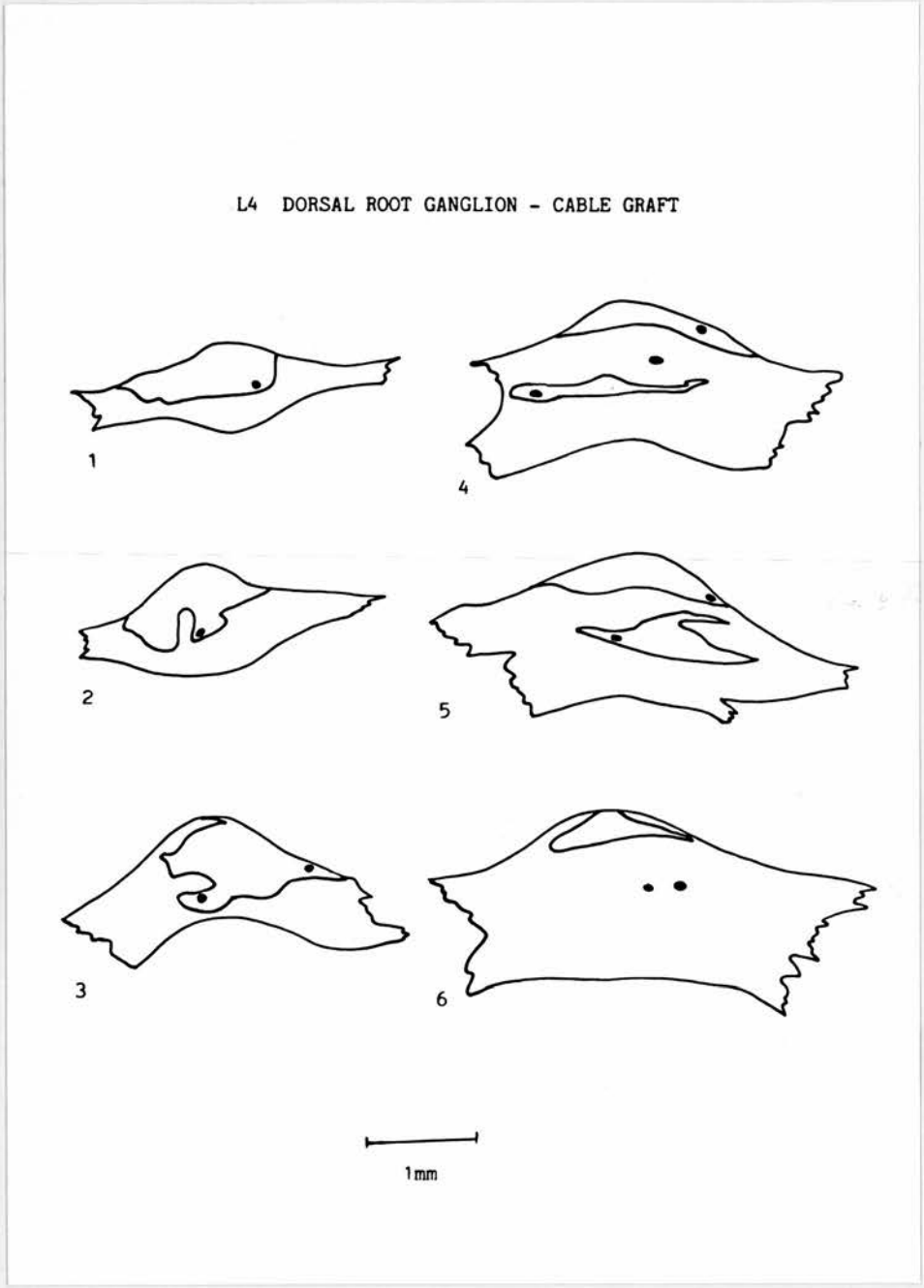

Figure 61: A diagrammatic representaion of 40µm serial sections of L4 DRG, 300 days after repair of the sciatic nerve by three-strand cable graft (continued overleaf).

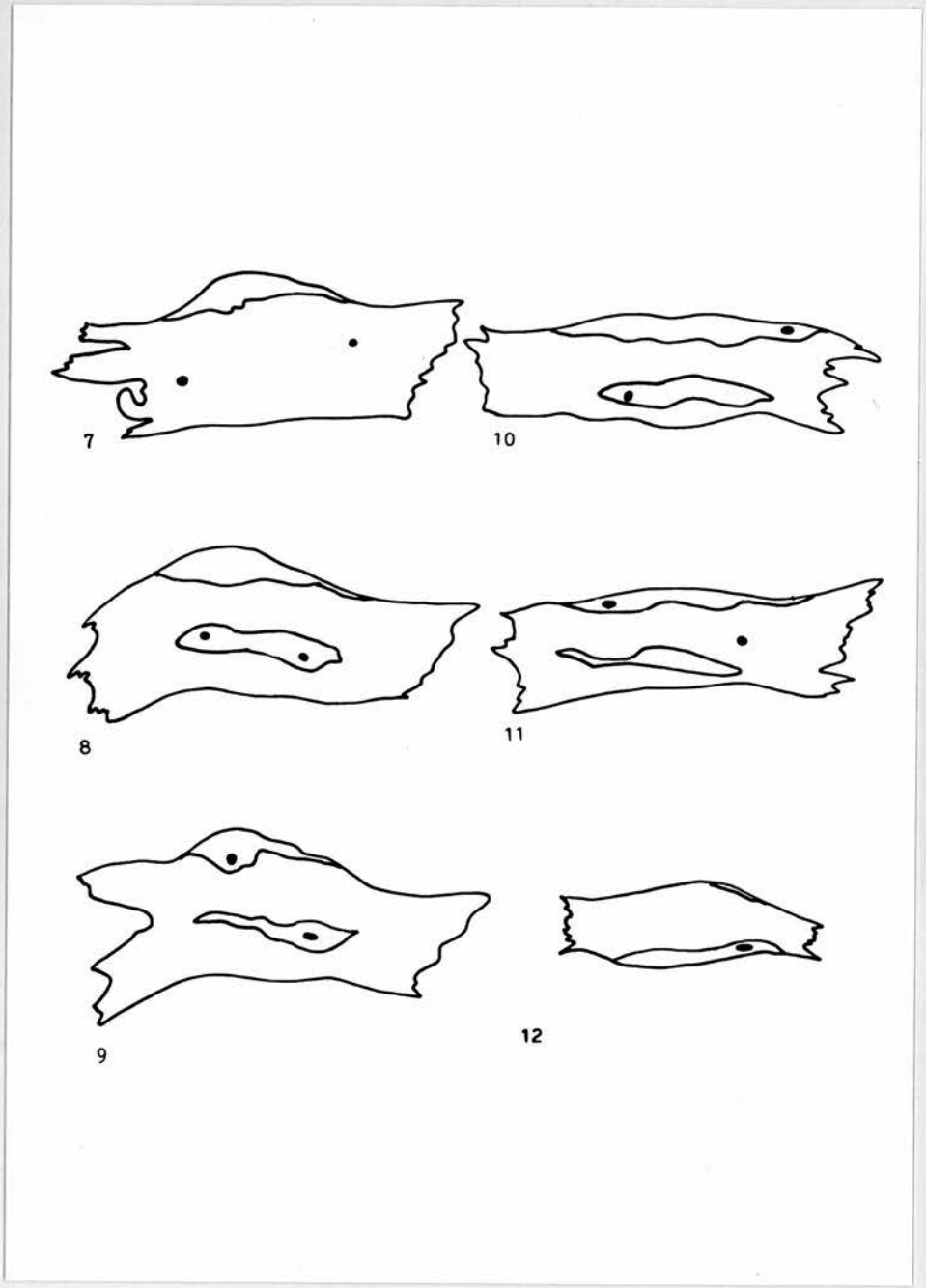

Figure 61: continued from previous page.

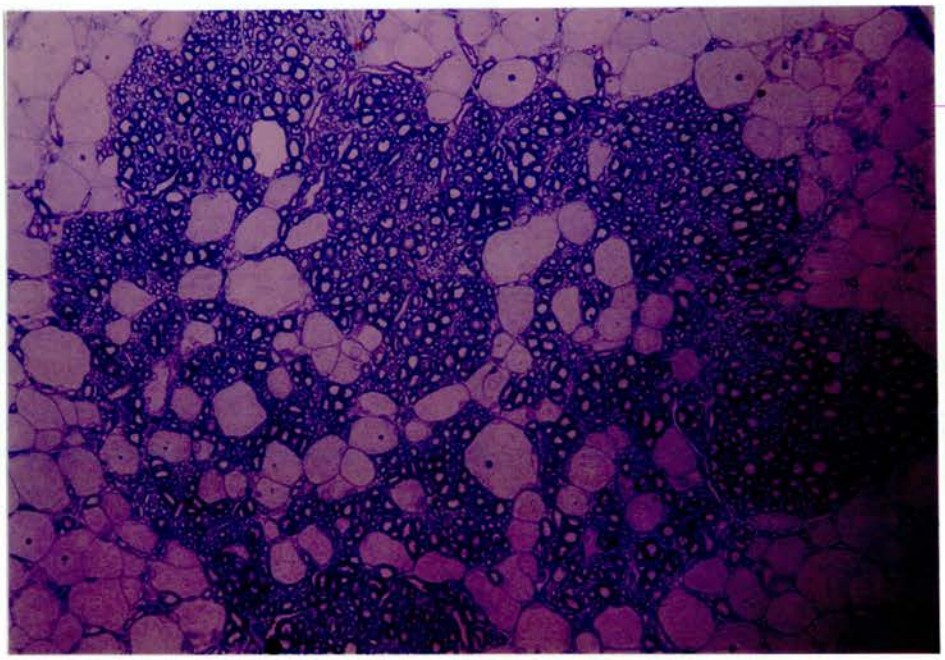

Figure 62: A semithin (1μm) plastic embedded, Toluidine Blue section of a normal L4 DRG showing the arrangement of the DRG cells within the ganglion. Irregular cords of cells lie interspersed with fibres. (x182)

4:4 Discussion

4:4:1 Cell Counts

After any of the three methods of repair undertaken in this study the total cell counts were significantly reduced from normal. Normal cell counts (113 ± 4) compare well with those made by Peyronnard, Charron, Lavoie *et al*, (1986a, 95 ± 10). Peyronnard *et al* used a different method of HRP application. The nerves to EDL were dissected free and the cut ends dipped into capillary tubes full of HRP solution. There is a greater chance of underestimating cells by this method as it is easy to miss the very fine nerve fibres which often pass to this muscle.

The percentage cell loss (29%) after nerve section and repair by epineurial suture found here compares well with that found after immediate repair of adult rat sural nerve by epineurial suture (30.6% at 280 days, Peyronnard, Charron, Lavoie *et al*, 1988). Interestingly this value is in the mid-range of values reported in Table 4:2 where no repair was undertaken.

Cell loss after muscle graft and cable graft repairs was greater, at 36% of normal, but the difference between these and direct N-N repair did not reach statistical significance. It would therefore appear that the degree of cell loss is related to the process of neurotomy and degeneration rather than to whether successful regeneration takes place, confirming Ranson's original statement (1906).

There has been some debate in the literature as to

whether the HRP method gives accurate cell counts. It has been stated that the HRP method underestimated the motorneurone pool (Peyronnard and Charron, 1983) and figures of 70% were found compared to axon counts (Nicolopoulos-Stournaras and Iles, 1983). After neurotomy injured cells did not take up or transport protein tracers (like HRP) as well as normal nerves (Kristenson and Olson, 1973,1975; Peyronnard, Charron, Lavoie *et al*, 1986b), presumably due to changes in the transport mechanisms within the neurone following axotomy. This failure of uptake and transportation of HRP continued as long as regeneration was prevented (Peyronnard, Charron, Lavoie *et al*, 1986b) and was particularly severe in primary sensory neurones of large diameter (Peyronnard, Charron, Lavoie *et al*, 1988). However it was shown that reanastamosing neurotomised nerves to their own distal segment delayed the decrease in HRP labelling of DRG neurones and sustained the uptake of HRP by many large cells for a long period of time (up to 80 weeks). After crush injury HRP labelling returned to normal values within 10 weeks (Peyronnard, Charron, Lavoie *et al*, 1988).

It would seem reasonable therefore to assume that HRP labelling 300 days after direct nerve suture and grafting methods will be of sufficient degree to allow accurate cell counts to be made.

4:1:2 Cell Diameter

Normal DRG cells showed a unimodal size distribution

(Figure 54). Cell diameters ranged from 14μm to 80μm with an average of 42.29μm. This is in keeping with the findings of other authors (Janig and McLachlan, 1984; Peyronnard, Charron, Lavoie *et al*, 1986a,1988)

Repair by all three methods was followed by a reduction in cell size which was significantly different from normal (Table 4:4). This has been noted by many authors and is due to i) loss of large cells and ii) atrophy of remaining cells (Carlson, Lais and Dyck, 1979; Janig and McLachlan, 1984; Rich, Luszczynski, Osborne *et al*, 1987; Peyronnard, Charron, Lavoie *et al*, 1988). Muscle and cable grafted nerve cell diameters were not significantly different from each other but both were significantly different from N-N suture. The degree of atrophy of any neurone is said to be dependent on whether or not appropriate peripheral connections have been made by that neurone (Aitken, Sharman and Young, 1947; Cavanaugh, 1951). This would explain why neurones repaired by direct nerve suture experience less atrophy than those repaired by grafts since the presence of two suture lines in grafted nerves increases the probability of mismatch between axons and appropriate endoneurial tubes. It is encouraging that there is no difference in this respect between cable grafts and muscle grafts. When the cable graft was inserted every effort was made to align peroneal fascicles with peroneal and tibial with tibial using individual cable strands (see chapter 2 for details). There is no opportunity to do this with a muscle graft. It would appear to make little

difference in this case. Presumably intrafascicular plexus disorganisation was unaffected by aligning major fascicles and obviously remained a major cause of mismatch of axons with end organs.

Ranson (1909,1912) stated that the size of the DRG cell reflected the size and type of axon associated with it. Large cells had large myelinated axons while small cells were associated with small myelinated and unmyelinated axons. Muscle-spindle and tendon organ afferents are large diameter myelinated fibres (Table 4:1). Loss and atrophy of the largest diameter DRG cells after nerve repair is presumably associated with loss of these large diameter fibres which may explain the lack of coordination seen after nerve injury. This will be investigated further in chapter 5.

4:1:3 Distribution of Stained Cells

Somatotopic organisation is well recognised in the sensory cortex of mammalian brain and we are all familiar with the pictures of homunculi representing this (Penfield and Rasmussen, 1950). This somatotopic representation of sensory modalities is also found in the dorsal horn of the spinal cord, the first relay station for cutaneous afferents (Devor and Wall, 1978,1981; Brushart, Henry and Mesulam, 1981).

In the normal rat, peroneal innervated hindlimb muscle afferents were found to project to a sharply defined area of the ipsilateral dorsal horn (Rexed's lamina II and III) at L3 segmental level. Skin afferents

projected to a wide area of the substantia gelatinosa, overlapping the area labelled from muscle. Nerve section and repair caused the muscle afferent projection to expand dramatically within the substantia gelatinosa and to extend caudally into the L4 segment (Brushart, Henry and Mesulam, 1981). The substantia gelatinosa of the dorsal horn receives the terminations of unmyelinated C fibres and Aδ fibres (see Table 4:1) from the dorsal root and these fibres are known to respond to noxious stimuli (Gobel and Falls, 1979; Ralston and Ralston, 1979).

In the cat, gastrocnemius-soleus muscle afferents were found to terminate in two separate groups:

i) In lamina VI-VII and Clarke's column. These are thought to represent the termination of large diameter afferents from muscle spindles and tendon organs.

ii) In lamina I and V. These are thought to represent the termination of small diameter nociceptive fibres (Mense and Craig, 1988).

A certain amount of evidence has accumulated from microelectrode studies of DRG cells to indicate that the intraganglionic portions of the sensory ganglion cell may not be completely irrelevant in determining the sequencing and patterning of peripherally generated impulses. There has been shown to be a passive influence in the form of a delay in the transmission of impulses through the ganglion (Dunn, 1955). Also, studies have revealed active properties of the ganglion cells under certain circumstances. Action potentials

can be generated by the cell body (Tagini and Camino, 1973; Kirk, 1974) and the soma is always invaded by the action potential as it passes along its processes (Letbetter and Willis, 1969).

If cells belonging to similar sensory modalities or if cells grouped together anatomically could be shown to have a distinct arrangement within the DRG this would add more weight to the idea that the DRG cells can influence the pattern of peripheral activity in some way. This has been shown to be the case for cutaneous afferents (Burton and McFarlane, 1973, as described in section 4:1) but information on muscle afferents has prevously been lacking. Peyronnard, Charron, Lavoie *et al*, (1986a) commented on a degree of clustering of muscle afferents in the DRG of the rat but found this to be inconsistent.

In this study, in the normal rat, afferents from EDL muscle were found to be clustered together in the DRG (Figure 58). This was found in all five rats studied suggesting that there is a degree of sensory organisation to be found. The arrangement of other normal hindlimb muscle afferents eg. tibialis anterior and soleus, need to be examined to see if there is a true somatosensory distribution across the ganglion as a whole.

After all three methods of nerve repair the localised arrangement of cells was lost. This was thought to reflect axonal mismatch during regeneration.

At a segmental level, normal EDL afferents were found

predominantly in L4 and L5 DRG, with occasional cells in L3 and L6. Stained cells were only found at three levels in any one rat (L3,L4 and L5 or L4,L5 and L6). This agrees with the findings of Peyronnard, Charron, Lavoie *et al*, (1986a). After nerve repair cells were found throughout L3-L6 DRG in all rats. The distribution of cells was widest in nerves repaired by cable and muscle grafts, with no apparent difference between them (Figure 52).

Thus it would seem that there are two levels of disorganisation seen after peripheral nerve repair, one at a segmental level and one at the level of the DRG itself. Disorganisation at a segmental level could be assumed to lead to uncoordinated and inappropriate spinal reflex responses while disorganisation within the DRG would lead to loss of any subtle facilitatory or patterning responses generated between cells at that level. Both levels of disorganisation would be reflected in uncoordinated motor control, due to loss of normal muscle stretch reflexes and, abnormal sensory appreciation.

Again it should be noted that no differences could be found between cable grafted and muscle grafted rats, despite the cable graft having a theoretical advantage over the muscle graft, in that more precise alignment of nerve fascicles is possible with a cable graft. This underlines the importance of intrafascicular disorganisation in determining the outcome after nerve repair.

CHAPTER FIVE

THE MOTOR REINNERVATION OF MUSCLE SPINDLES

5:1 The muscle spindle is a unique sense organ. It has both a sensory and a motor nerve supply. It has been shown in Chapter Four that the sensory nerve supply to muscle was significantly disordered, at several levels, after nerve repair. This chapter aims to look at the motor supply of muscle spindles after nerve repair.

5:2 The Normal Muscle Spindle

The muscle spindle is a specialisation of skeletal muscle cells (see Matthews, 1972 and Hulliger, 1984 for reviews of muscle spindle anatomy and physiology).

In brief, there are two types of spindle (intrafusal) muscle fibres, namely bag and chain fibres. Bag fibres are so named because of the bag-like swelling in the centre of the cell which contains a collection of nuclei. Chain fibres are long and thin and their nuclei are arranged in chains throughout the length of the fibre (Figure 63). There are two kinds of afferent fibres associated with the spindle. These are the type Ia and type II fibres investigated in Chapter Four. The spindle also has a motor supply which can be divided into two groups. The gamma fibre nerve supply is motor to intrafusal fibres only while the beta fibres are branches of alpha motor neurones.

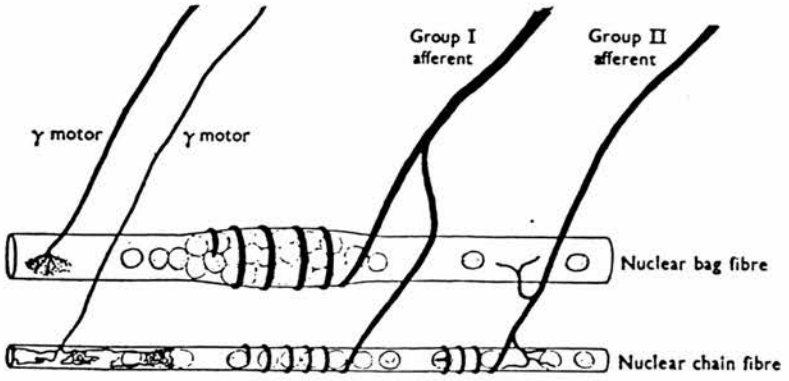

Figure 63: Diagram of a muscle spindle

Like skeletal muscle fibres, spindles were found to undergo atrophy after nerve section and to regenerate as the muscle became reinnervated (Ducker, 1972). Nerve fibres did not selectively reinnervate their own muscle fibres (Weiss and Hoag, 1946; Scherer, 1986). Sensory nerve fibres have been shown to make contact with muscle fibres but failed to form functional connections and were unable to reverse the atrophy associated with denervation (Zalewski, 1970). There can be little doubt that spindle function after nerve regeneration, as measured by afferent responses to stretch, is significantly disordered (Hyde and Scott, 1983; Banks and Barker, 1989) however this could be due to end organ atrophy as well as afferent fibre disorganisation. If the motor nerve supply to muscle spindles failed to regain contact completely then the spindle would remain atrophic and would eventually disappear for the reasons discussed above. If only partial or inappropriate motor reinnervation took place and the spindle failed to regain its full size then this perhaps could contribute

to the functional changes found in spindles after nerve repair. It was decided to approach the problem by firstly attempting absolute spindle counts in the EDL muscle before and after nerve section and repair.

5:3 Materials and Methods

Four groups of rats were studied. There were five rats in each group. In one group of normal rats the left EDL muscle was denervated completely by sectioning the peroneal nerve at the knee. The ends of the nerve were tied with 3/0 silk suture to prevent regeneration. These rats were left for eight weeks to allow complete muscle atrophy to occur.

Three other groups of rats in which the sciatic nerve had been repaired 300 days previously by direct N-N suture, three-strand cable graft or muscle graft, as described in Chapter Two, were also investigated. The normal, contralateral EDL muscle was used as a control.

Serial, paraffin embedded, Haematoxylin and Eosin stained sections of EDL muscle were used as each muscle spindle was easily seen and could be followed in its entire length through successive sections using the light microscope.

5:4 Processing EDL Muscle for Paraffin Wax Sections.

See appendix for solutions

1) EDL muscle was removed from the anaesthetised rat (see Chapter Two) and pinned out to normal resting length on

dental wax. The muscle was left in a moist atmosphere covered with tissue paper soaked in 0.9% saline for 15-20 minutes to allow the muscle fibres to relax.

2) the muscle was fixed in 10% formal saline for 24 hours still pinned to the wax.

3) The muscle was removed from the wax and the ends were trimmed free of tendon. It was then immersed in 70% ethanol for two to four hours. It was dehydrated in diethylphosphate for 36 hours (three changes) following which it was cleared in benzene for 20-40 minutes.

4) The tissue was infiltrated with molten paraffin wax at 64°C (three x five minute changes under negative pressure).

5) The specimens were orientated in moulds and embedded in molten paraffin wax and cooled in running tap water. When hard the block was mounted onto a wooden block and cut serially at 9 µm on a Reichart-Jung microtome.

6) Glass slides were cleaned in detergent, rinsed in absolute ethanol then coated with a mixture of egg albumin and glycerine. The sections were mounted in water onto these slides and dried overnight at 37°C.

7) The sections were dewaxed in xylene (two x five minute

changes) and brought to water through a series of graded alcohols.

8) The sections were stained as follows:
Place in alum haematoxylin for 20 minutes.
Wash in running tap water.
Differentiate in acid alcohol for 5-10 seconds.
Wash in running tap water for at least five minutes or until there is a noticeable colour change.
Counter stain in eosin for four minutes.
Wash in running tap water until the water runs clear.

9) The sections were then dehydrated through a series of alcohols, cleared in xylene, coverslipped with DPX mountant and allowed to air dry for at least 24 hours.

Each section was scanned under the light microscope for muscle spindles which could be followed through successive sections. They were counted by hand.
In one rat of the denervated group there was evidence of muscle fibre recovery presumably due to reinnervation of the muscle. This rat was excluded for this reason.

5:5 Results

The four remaining rats in the total atrophy group showed extreme EDL muscle atrophy, as expected. In the other groups the EDL muscle was indistinguishable from normal in terms of muscle size. (This has already been confirmed in Chapter Three, see Figure 43 & Tables

3:17; 3:18) However, there was still evidence of muscle imbalance in these rats. Their gait was still abnormal and some had abnormal flexion of the toes on the affected side. The toe-spreading reflex had returned in all of the rats which had undergone nerve repair.

Table 5:1 shows the absolute muscle spindle counts in the total atrophy group compared to normal. The muscle showed typical atrophic changes, at a light microscope level, as was expected.

The counting error was zero in normal EDL but increased to 3.8% in grafted groups.

Table 5:1

	Number of Spindles	
Rat No.	Atrophic EDL	Normal EDL
1	3	28
2	5	24
3	6	31
4	5	30
5	excluded	26
Mean	4.75	27.8
SD	1.09	2.86
SEM	0.55	1.28

All spindles seen in the total atrophy group were severely shrunken and atrophic compared with normal. For this reason they were very difficult to identify. This is in keeping with the hypothesis outlined in

section 5:2.

Figure 64 shows spindle counts 300 days after repair of the sciatic nerve by various means. There were no significant differences between any of the groups or between repaired nerves and normal in terms of numbers of spindles seen after nerve regeneration.

Figure 65 shows a normal muscle spindle. Figures 66 and 67 show muscle spindles after nerve regeneration. After regeneration the spindles never regained the plump appearance of the intrafusal fibres seen in the normal spindle.

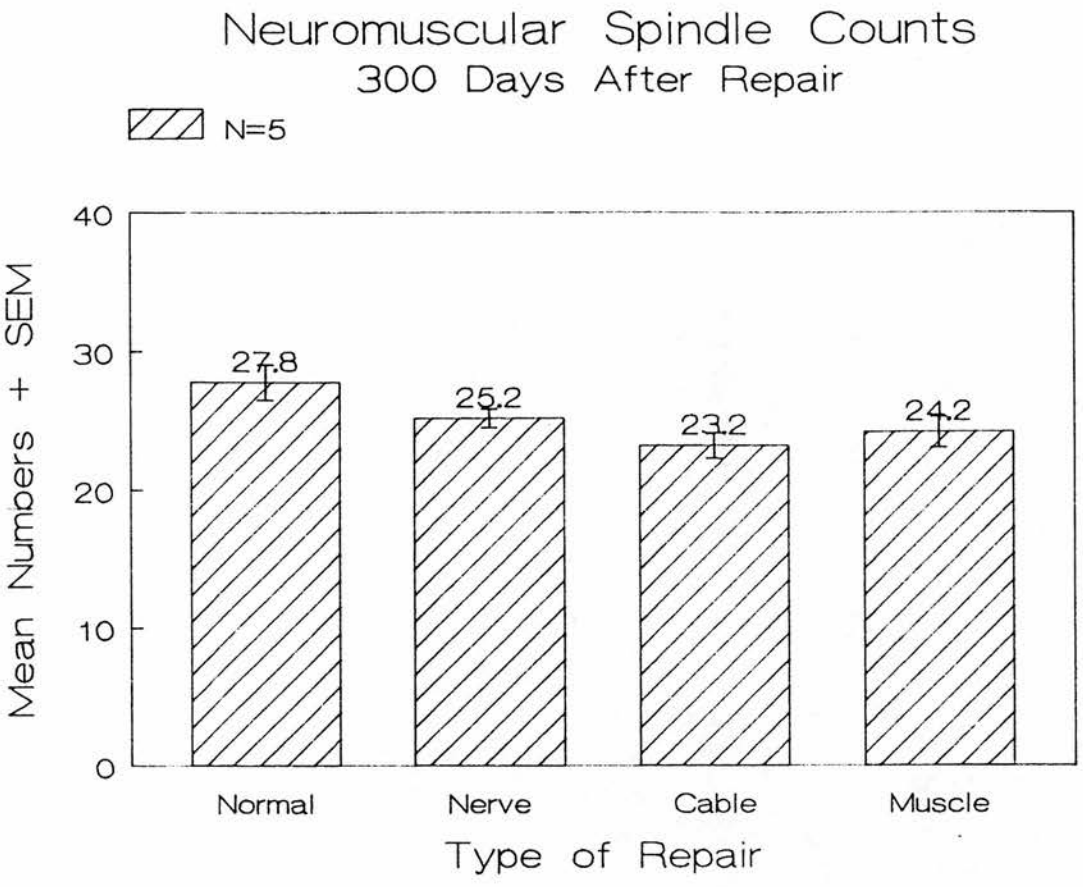

Figure 64: Numbers of neuromuscular spindles in EDL muscle 300 days after repair of the sciatic nerve.

Figure 65: Photograph of a 9µm haematoxylin & Eosin stained paraffin wax section of EDL muscle showing the normal appearance of a muscle spindle in transverse section. (x900)

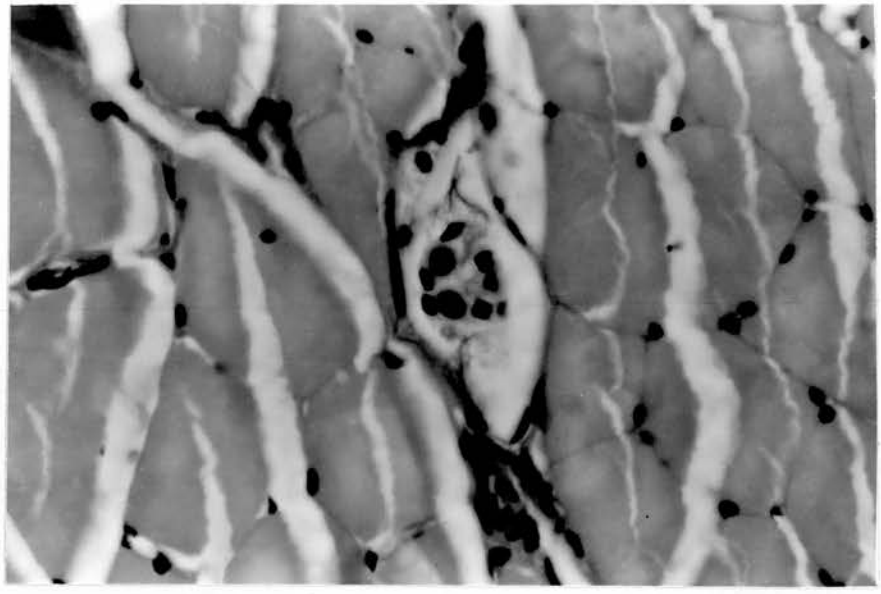

Figure 66: Photograph of a 9µm haematoxylin & Eosin stained paraffin wax section of EDL muscle showing the appearance of a muscle spindle in transverse section (x1250). The sciatic nerve had been repaired by direct nerve suture 300 days previously.

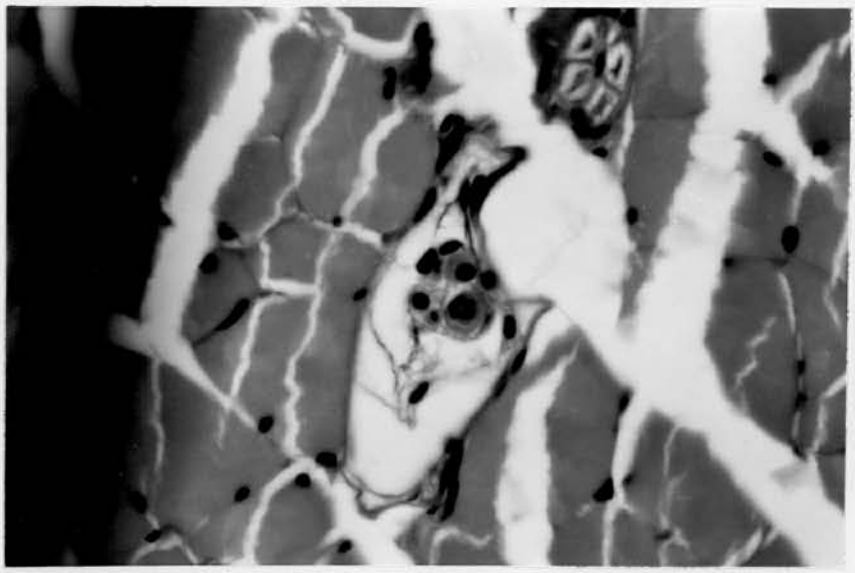

Figure 67: Photograph of a 9μm haematoxylin & Eosin stained paraffin wax section of EDL muscle showing the appearance of a muscle spindle in transverse section (x1250). The sciatic nerve had been repaired by muscle graft 300 days previously.

5:6 Discussion

Spindle counts after complete muscle denervation were very low confirming the observations of Ducker (1972). Normal spindle counts in EDL muscle compared well to spindle counts in mouse EDL (Personal communication from Dr Fanney Kristmunsdottir, 1990). During muscle reinnervation spindles were also reinnervated and recovered from their atrophied state. There were no significant differences in the numbers of spindles seen after nerve regeneration in any of the experimental groups and the numbers of spindles seen in these groups were not significantly different from normal. However the regenerated spindles did not recover their normal appearances, remaining smaller than normal. Regenerated spindles also took up the Haematoxylin and Eosin stain more avidly, staining more darkly than normal spindles. The intrafusal fibres of normal muscle spindles looked plump and rounded and this appearance was less marked in regenerated spindles. It was also much more difficult to distinguish between bag and chain fibres in regenerated spindles.

It would be interesting to obtain absolute measurements of the size of regenerated spindles to confirm the impressions given above but this was not possible in this preparation since the diameter of the spindles varied along their lengths to such a degree that comparisons would have been misleading.

It would seem from these findings that muscle spindles receive enough motor nerve supply after nerve

regeneration to reverse the atrophy seen after denervation and that loss of spindle numbers does not contribute to the disordered function seen after nerve repair. However the impression is that intrafusal fibres do not recover their normal morphology completely, remaining slightly smaller than normal. Also, this study did not take into account any differences in gamma and beta motor innervation. It is interesting that grafting techniques did no worse than direct nerve suture in these respects and that there were no significant differences between muscle and cable grafted groups.

CHAPTER SIX

CONCLUSIONS

Conclusions have been drawn at the end of each chapter and I have no intention of repeating these but some aspects of the work require further discussion. In particular a common theme running through most chapters in this thesis is the problems caused by the small numbers of animals used in each of the experimental groups. This was of course due to restraints placed on some occasions by the Home Office Inspector but in other cases simply due to the constraints of time and money. In most cases the numbers used were adequate to give a satisfactory answer but in a few cases it would be desirable to repeat the experiment with a greater number of animals.

I think it is important for scientific research to go hand in hand with clinical research since, especially in this case, many of the recognised techniques used to assess the recovery of grafted nerves are not available to the surgeon. The aims of this research were in the first instance to present absolute evidence to surgeons for the efficacy of the muscle graft in nerve repair in the rat. They could then go forward knowing that there was a firm scientific basis for the use of the muscle graft. Hopefully in the process I managed to throw a little light on the mechanism of nerve regeneration.

The role of the rat in peripheral nerve repair

research has been critisised because of its supposed superior regenerative capacity. Mackinnon and Trued (1986) are often quoted in defence of the rat as a suitable model for nerve repair and regeneration however the work of Jenq and Coggeshall (1986, 1987) is more convincing. They have shown that a one centimetre gap in the rat sciatic nerve is a significant lesion that would be unlikely to recover spontaneously without surgical intervention. For this reason I think it is now accepted by most people that the rat is a reasonably good model for nerve repair. It does however have the advantage of much faster recovery because of the smaller distances that axons have to regenerate (rat sciatic nerve is roughly 13 cm long compared to human sciatic nerve which can be over 1m in length).

There can be little doubt that when direct nerve suture is performed under optimal conditions with no tension it is superior to grafting techniques. However when a grafting technique is to be used this work has shown that in every case the muscle graft was as good as the conventional technique of cable grafting. In addition, it must be emphasised that the muscle graft has a few advantages over cable grafting in the clinical setting. Firstly, the technique of muscle grafting eliminates the need to harvest previously healthy nerves to furnish a graft, thus sparing the patient a second (or even third) incision with subsequent scarring and potential loss of sensation in the territory of the

donor nerve. Secondly, there is an abundant supply of skeletal muscle in the body and so one should never have to sacrifice a potentially salvageable nerve simply because of lack of appropriate grafting material. Thirdly, in most cases the muscle used to prepare the graft can be taken through the same incision as is used to expose the injured nerve *e.g.* the graft can be taken from semimembranosus muscle when repairing the common peroneal nerve, or from sartorius muscle when repairing the femoral nerve. For these reasons the technique of muscle grafting should now be accepted as a method of peripheral nerve repair in the human when the defect cannot be repaired directly and a graft of some description is necessary.

This work has also emphasised that no matter how well the graft is aligned or how good the technique, intrafascicular disorganisation will occur to some degree and unfortunately this will never be amenable to surgical correction. I think the way forward in nerve repair will be to look more closely at methods designed to improve the number of appropriate connections made. A better understanding of the ways in which tissues interact with each other will be required before this will be in our grasp.

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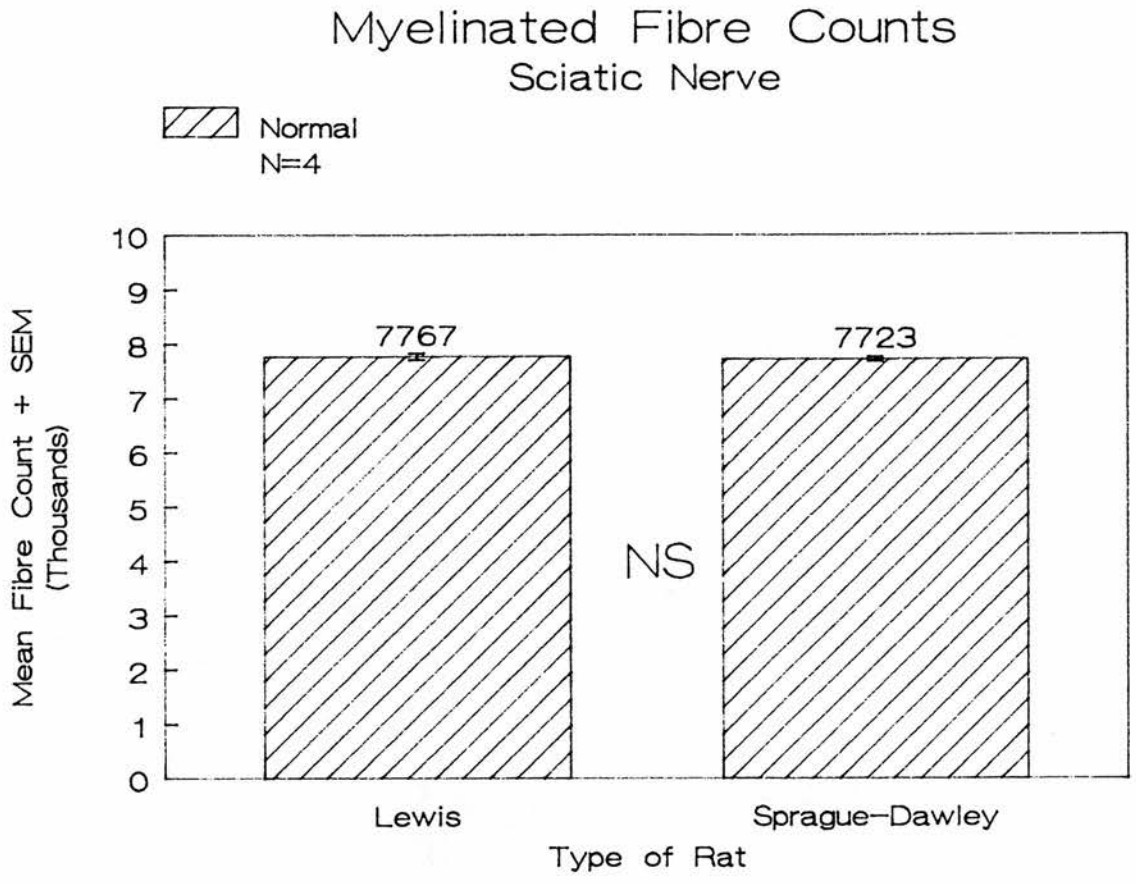
APPENDIX ONE

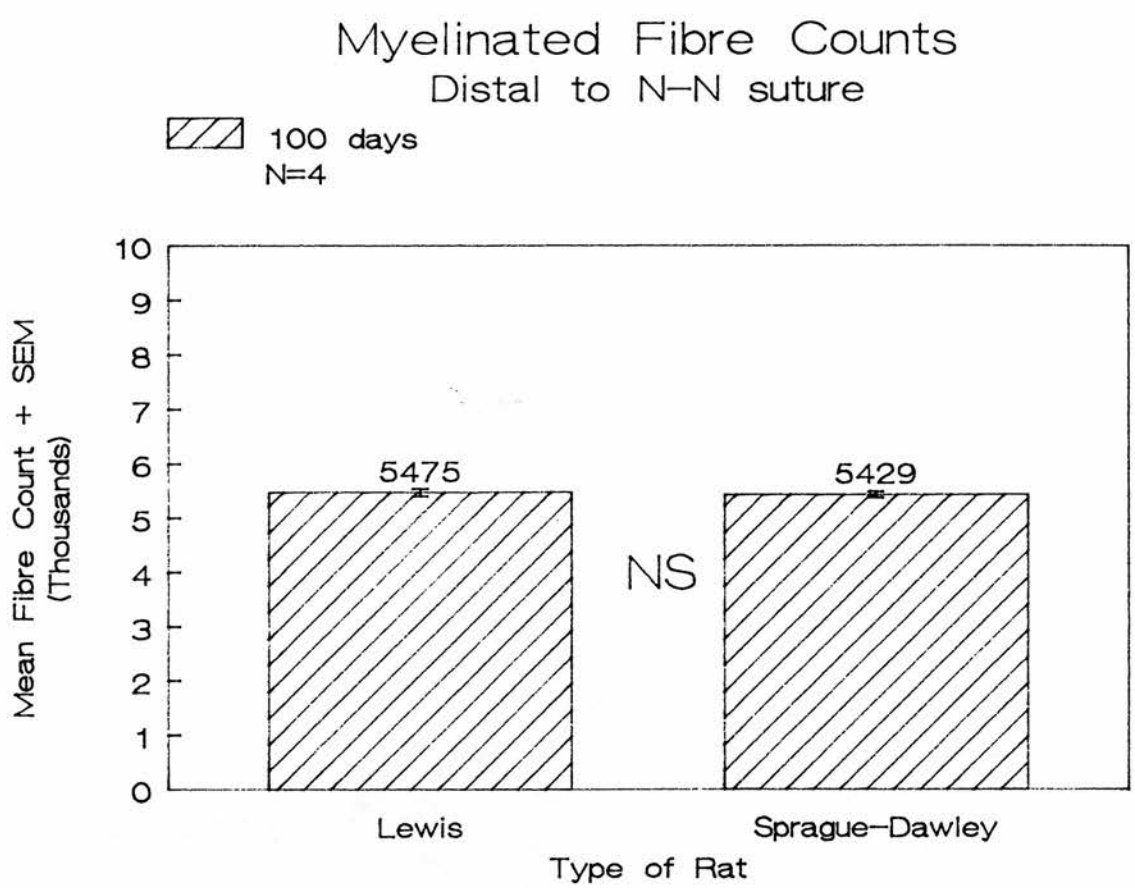
Lewis Rats Compared to Sprague-Dawley Rats.

a) Table of Physiological Variables. (N=4)

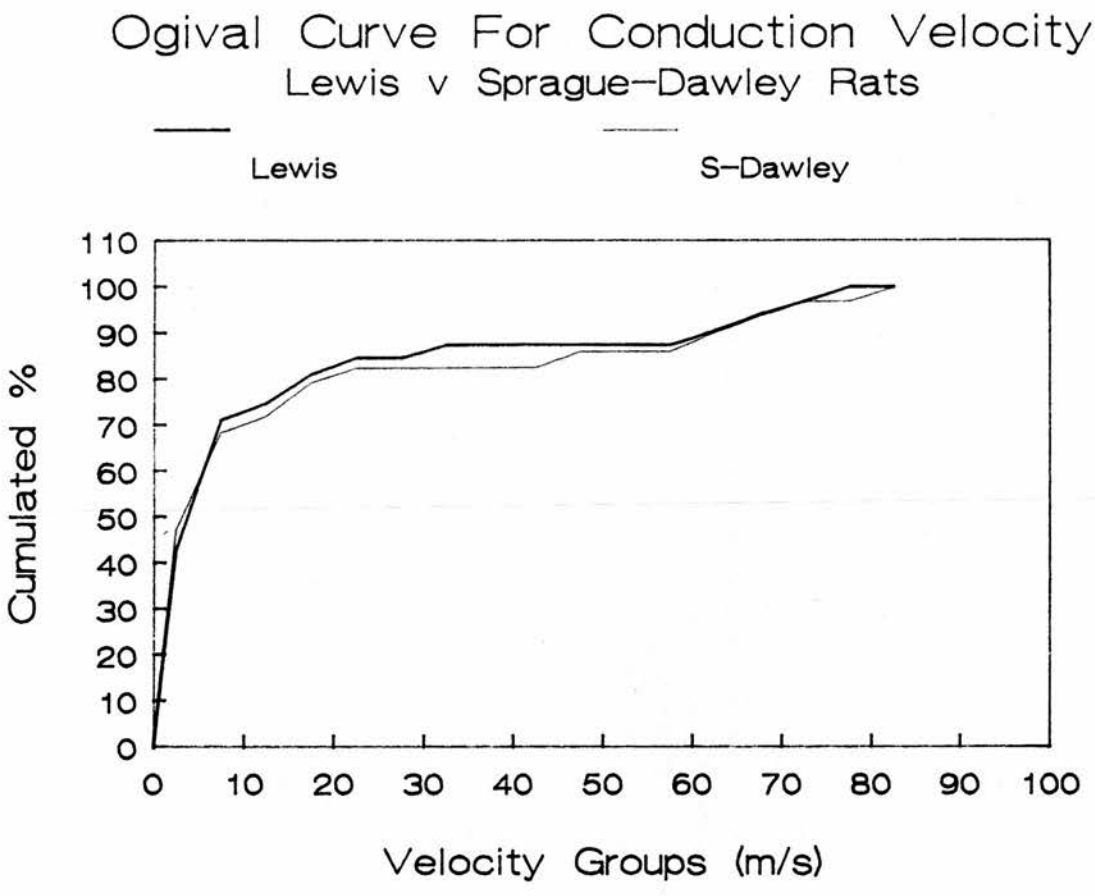
VARIABLE	SD RATS	P-VALUE	LEWIS RATS
Area Iso	$\bar{x}$=0.0243		$\bar{x}$=0.0298
-Myo.	SD=0.0080	NS	SD=0.0067
(Ns)	SEM=0.0040		SEM=0.0034
Peak Iso	$\bar{x}$=0.5205		$\bar{x}$=0.4878
-Myo.	SD=0.2126	NS	SD=0.2066
(N)	SEM=0.1063		SEM=0.1033
Time to	$\bar{x}$=22.125		$\bar{x}$=20.700
Peak.	SD=2.8020	NS	SD=3.6020
(msec)	SEM=1.4008		SEM=1.8009
Durn.of	$\bar{x}$=98.675		$\bar{x}$=101.75
Twitch	SD=3.3140	NS	SD=9.0648
(msec)	SEM=1.6570		SEM=4.5320
Area of	$\bar{x}$=214.55		$\bar{x}$=220.8
Int.EMG	SD=47.143	NS	SD=27.270
(mVs)	SEM=23.572		SEM=13.635
Motor	$\bar{x}$=15.990		$\bar{x}$=16.845
Latency	SD=4.915	NS	SD=5.515
(ms^{-1})	SEM=2.457		SEM=2.758



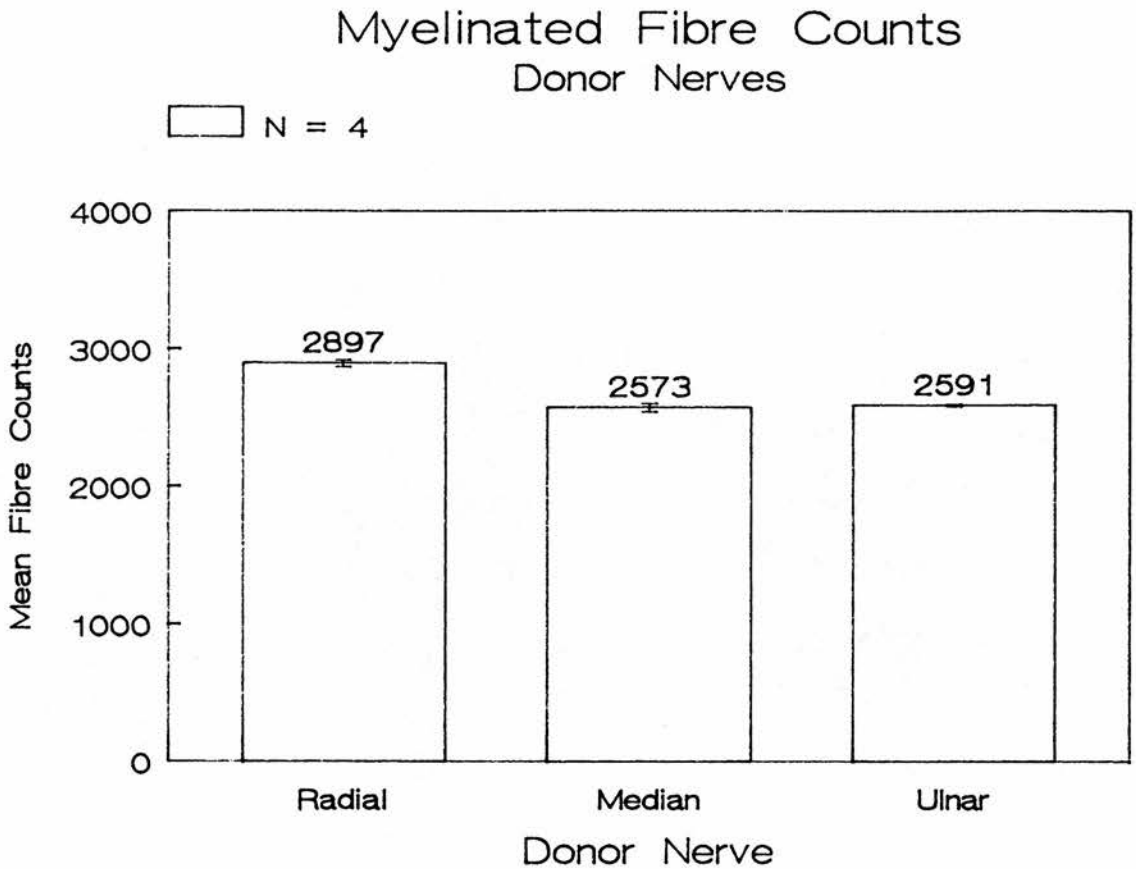
Absolute fibre counts are not significantly different between the two groups of rats (shown above).



Regenerating fibre counts in the distal nerve after a N-N suture are not significantly different, after 100 days, showing that the regenerative response is similar in both groups.



Conduction velocities are very similar as shown above.



Myelinated Fibre Counts in Donor Nerves

APPENDIX THREE

Information on Fibrin Glue*

Tisseel*

Made by: Immuno Ltd,
Artic House,
Dunstane Green,
Nr Sevenoaks,
KENT

Tisseel is a fibrin glue made from pooled human plasma. It consists of two components which have to be mixed before use.

1) Fibrinogen solution. 1ml contains:
Fibrinogen 70-110mg
Plasma Fibronectin 2-9mg
Factor XIII 10-50µg
Plasminogen 40-120µg
100KIU of Aprotinin solution is added to this (protease inhibitor).

2) Thrombin solution. 4IU/ml
plus 1ml of 40 mmol $CaCl_2$/l (activator).
These solutions are mixed and applied using the supplied "Duploject" system which allows simultaneous application of equal amounts of the two components. The glue sets in 30-60 seconds.

APPENDIX FOUR

Solutions for processing semithin sections.

1) 0.4M SODIUM CACODYLATE

Dissolve 8.5612g sodium cacodylate in 50ml of distilled water. Make up to 100ml with distilled water.

2) 0.2M SODIUM CACODYLATE BUFFER at pH 7.4

Take 50ml of 0.4M sodium cacodylate. Use 0.2M HCl (approx. 8ml) to pH the solution to 7.4. Make up to 100ml with distilled water.

3) 0.1M SODIUM CACODYLATE BUFFER AT pH 7.4 Take 50ml of 0.2M sodium cacodylate buffer and make up to 100ml with distilled water.

4) 4% GLUTERALDEHYDE in 0.1M SODIUM CACODYLATE BUFFER

Take 50ml of 0.2M sodium cacodylate buffer at pH 7.4. Add 16ml of 25% glutaraldehyde and pH to 7.4 again. Make up to 100 ml with distilled water.

5) 10% SUCROSE BUFFER

Take 50ml of 0.2M sodium cacodylate buffer: Put on to stir, add 10g of sucrose <u>while stirring</u>. ph to 7.4 again and make up to 100ml with distilled water.

6) 0.1% SOLUTION of OSMIUM TETROXIDE in 0.1M SODIUM CACODYLATE BUFFER

Break a 0.1g ampoule of osmium tetroxide in a universal container with the lid screwed on, in a fume cupboard. Add 10ml of 0.1M sodium cacodylate buffer at pH 7.4 to the bottle. Make sure the cap is screwed on tightly and mix carefully. Leave overnight to allow the solution to mix fully. Can be stored in a freezer at -4°C.

APPENDIX FIVE

Normal unoperated rats compared to the normal side after nerve repair.

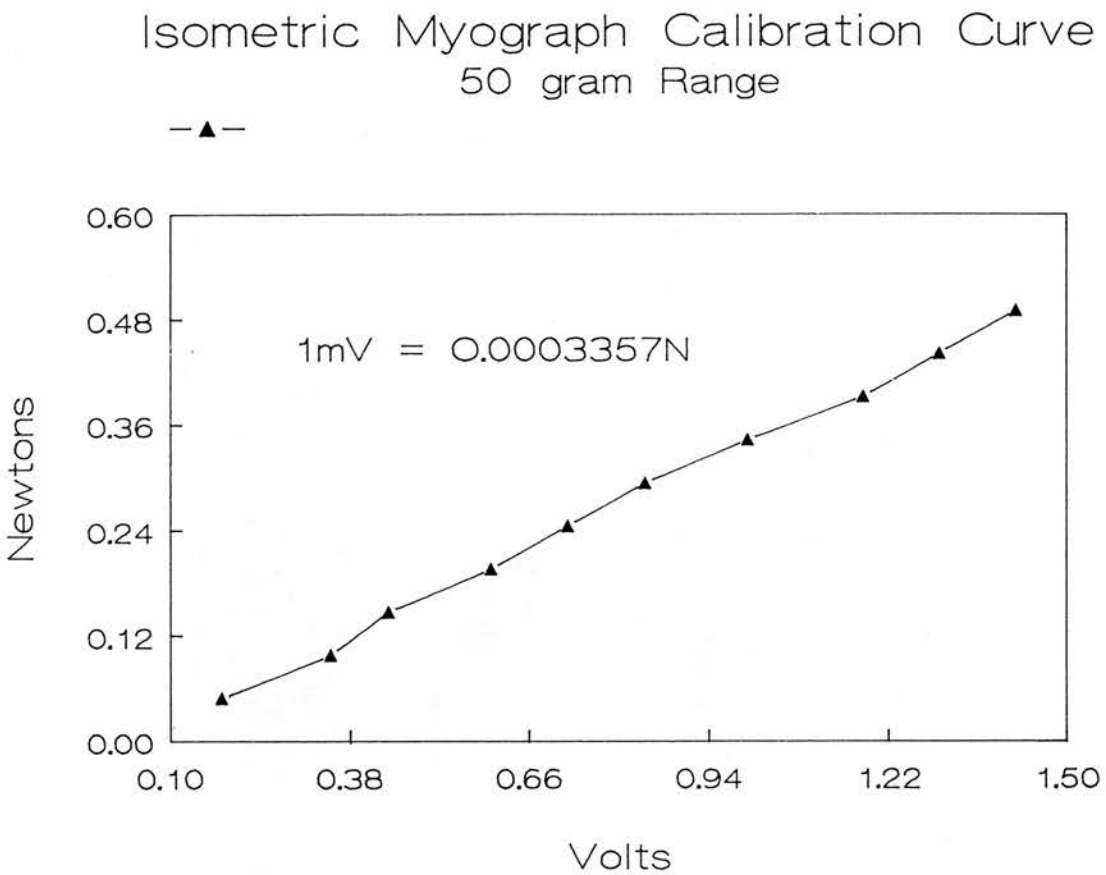
MUSCLE WEIGHTS (g)

Normal Unoperated	Normal Cable	Normal Muscle	Normal N-N
N=4	N=4	N=4	N=4
x=0.2509	x=0.2470	x=0.2395	x=0.2470
SD=0.0339	SD=0.0146	SD= 0.0233	SD=0.0214
SEM=0.0169	SEM=0.0073	SEM=0.0116	SEM=0.0107

MAXIMUM TWITCH (N)

N=4	N=4	N=4	N=4
x=0.600	x=0.732	x=0.669	x=0.693
SD=0.0955	SD=0.1541	SD=0.0892	SD=0.1474
SEM=0.0478	SEM=0.0771	SEM=0.0446	SEM=0.0737

No comparison reaches statistical significance.



Isometric Myograph Calibration Curve

APPENDIX SEVEN

HRP Method Solutions.

1) PHOSPHATE BUFFER SOLUTION (0.2M at pH 7.4) Dissolve 7.8g of monobasic sodium phosphate ($NaH_2PO_4-H_2O$) in 250 ml of distilled water. In a separate beaker dissolve 32.5g of dibasic sodium phosphate (Na_2HPO_4) IN 1000ml of distilled water. Mix the two solutions to obtain 1250ml of 0.2M phosphate buffer at pH 7.4.

2) PHOSPHATE BUFFERED SALINE

Dissolve 7.6g of dipotassium hydrogen orthophosphate, 1.3g of sodium dihydrogen orthophosphate and 7.2g of sodium chloride in 750ml of distilled water. Make up to 1000ml with distilled water.

3) ACETATE BUFFER AT pH 3.3

Mix 100ml of 1M sodium acetate, 100ml of distilled water and 95ml of 1M hydrochloric acid. Make up to 500ml with distilled water. Check the pH. Titrate with concentrated acetic acid or sodium hydroxide in order to bring the pH to 3.3. This will keep for one week at 4°C.

4) TETRAMETHYLBENZIDINE INCUBATION SOLUTION (TMB)

Solution A: Mix 187.5 ml of distilled water, 10ml of acetate buffer at pH 3.3 and 200mg of sodium nitroferrycyanide.

Solution B: Add 10mg of 3,3',5,5' teramethylbenzidine to 2.5ml of absolute ethanol. Heat to 40°C to dissolve the TMB.

Mix solutions A and B immediately before use to obtain

the incubation solution. A+B must be less than two hours old.

5) 0.3% HYDROGEN PEROXIDE SOLUTION

Add 1ml of a 30% solution of hydrogen peroxide to 100ml of distilled water immediately before use.

6) FIXATIVE FOR PERFUSION

Dissolve 10g of paraformaldehyde in 100ml of distilled water at 70°C.Add drops of 1N NaOH until the solution clears. Cool to 40°C. Add 50ml of 25% glutaraldehyde and make up to 500ml with distilled water. Add 500ml of 0.2M phosphate buffer.

7) SUCROSE BUFFER

Add 10g of sucrose to 100ml of 0.2Mphosphate buffer while stirring.

8) HRP SOLUTION FOR INJECTING

Add 20mg of horseradish peroxidase enzyme (Sigma) to 100ul of 0.9% saline and shake to dissolve.

APPENDIX EIGHT

Solutions for processing EDL muscle in Paraffin Wax

1) 10% FORMAL SALINE

10 ml of formaldehyde made up to 100 ml with normal saline.

2) NORMAL SALINE (0.9% sodium chloride solution)

Dissolve 9g of sodium chloride in 1000ml of distilled water.

3) GRADED ALCOHOL SERIES

100% Ethanol, 45 seconds. (minimum times)

100% Ethanol, 45 seconds.

96% Ethanol, 45 seconds.

96% Ethanol, 45 seconds.

70% Ethanol, 60 seconds.

Distilled Water

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DEPARTMENT OF ANATOMY
Professor M.H. Kaufman

UNIVERSITY MEDICAL SCHOOL,
TEVIOT PLACE,
EDINBURGH.
EH8 9AG

15th August 1990

Issei Seyama

Professor ~~Yoshiyasu Matsuo~~
Hiroshima University School of Medicine
Hiroshima Daigaku Igakubu
1-2-3 Kasumi
Hiroshima 734
JAPAN

Seyama

Dear Professor ~~Matsuo~~,

I am preparing a thesis entitled 'The Muscle Graft - A New Method of Peripheral Nerve Repair', which will be submitted to Edinburgh University as an MD thesis. I should be grateful if you would grant permission for the following to be reproduced:

Diagram indicating relationship between tension at suture site and stage of circulatory disturbance on proximal side of the nerve trunk, in Miyamoto, Y.: Experimental studies on repair of peripheral nerves: relationship between circulatory disturbance at nerve stumps caused by tension at suture line and axon regeneration. Hiroshima J. Med. Sci, 28:87 (1979).

The usual acknowledgements and a full reference to the paper will of course be included. If you would like the credit line to take any special form, please let me know what this should be.

Would you please indicate your agreement by signing and returning one copy of this letter. Thank you for your co-operation.

Yours sincerely,

(Dr) Lynn Myles

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APPENDIX TEN

PAPERS AND PRESENTATIONS OF THIS WORK

Parts of this thesis were presented to the Royal College of Surgeons of Edinburgh, as the Lister Lecture, on the 7th December 1990. I was also awarded the Lister Professorship 1990 for this work.
Published papers are bound overleaf.

British Journal of Plastic Surgery (1990), **43**, 169–178
© 1990 The Trustees of British Association of Plastic Surgeons
0007–1226/90/0043–0169/$10.00

The repair of large peripheral nerves using skeletal muscle autografts: a comparison with cable grafts in the sheep femoral nerve

M. A. GLASBY, J. A. GILMOUR, S. E. GSCHMEISSNER, T. E. J. HEMS and L. M. MYLES

Department of Anatomy, University of Edinburgh Medical School

Summary—Recovery of morphological characteristics was compared in the femoral nerves of sheep at varying times up to 10 months after nerve repair. Groups of sheep receiving coaxially aligned freeze-thawed skeletal muscle autografts were compared with those receiving three-strand cable grafts made from autogenous cutaneous nerve. At all times after implantation more nerve fibres could be counted distal to the muscle grafts than distal to cable grafts. Indices of nerve fibre maturation were indistinguishable between the two groups at 10 months. The results are discussed in relation to the possible use of the technique for repairing large mixed nerves.

After transection of a peripheral nerve, end-to-end suture is possible only if the length of damaged or lost nerve is very short. Suture of nerves under tension produces poor results (Terzis *et al.*, 1975; Mackinnon and Dellon, 1988) and nerve autografts have been used to fill such defects. However recent work (Glasby *et al.*, 1986a, b, c, d, 1988a, b; Davies *et al.*, 1987; Gattuso *et al.*, 1988a, b, 1989; Norris *et al.*, 1988; Glasby, 1990) has shown coaxially aligned freeze-thawed muscle autografts to be a satisfactory means of repair in nerve defects of various species. Recovery in these cases was assessed variously by physiological and anatomical comparison with the contralateral normal nerve, direct end-to-end suture and equal diameter nerve grafts.

The repair of large diameter human nerves poses a particularly difficult problem (Seddon, 1954, 1975; Sunderland, 1978; Birch *et al.*, 1986; Mackinnon and Dellon, 1988), as it is unlikely that a donor autograft of equal diameter will be available. A cable graft, *e.g.* sural nerve, thus offers the only currently feasible treatment with a number of strands (constituting a cable) used to achieve a diameter equivalent to that of the host nerve. In the case of a long defect, the intervention necessary to obtain such a length of nerve may be considerable. Inevitably there is some mismatch of fasciculi at both ends of the graft, resulting in loss of fibres.

Cable grafting may offer an advantage over a single trunk graft in that fasciculi at the proximal end can be connected to appropriate fasciculi in the distal stump (Millesi *et al.*, 1972; Mackinnon and Dellon, 1988). However, this requires separation of fasciculi which is likely to cause further tissue damage, introduces additional foreign material and may increase the infiltration of connective tissue into the site. Fascicular anatomy changes frequently along nerves (Sunderland, 1978), which makes correct "rewiring" difficult. In addition it is most unlikely that the fascicular pattern in the donor nerve will match the pattern of the host nerve.

Cable grafts suffer from ischaemia and formation of fibrous connective tissue between strands. Muscle grafts can be placed in one block cut to any size, (Glasby *et al.*, 1986c, d; Norris *et al.*, 1988; Glasby, 1990) and may not rely on the viability of the cells present in them (Fawcett and Keynes, 1986).

The early clinical use of freeze-thawed coaxially aligned skeletal muscle autografts in the repair of human digital nerves has been reported by Norris *et al.* (1988) and Glasby (1990). Early impressions are of a technically simple procedure which compares favourably with other techniques. Cracking of the graft during thawing is sometimes a problem and it is necessary to anticipate a shrinkage in the length of the graft by up to 50%. A number of cases of the repair of larger nerves have been undertaken and results are awaited. An ethical problem arises in the extension of the technique to the repair of large nerves without the prior comparison with cable grafts which, though far from ideal, represent the established treatment for such cases. At present

muscle grafts are restricted on ethical grounds to those previously inoperable cases where the required size of the cable would exceed the available graft material. Theoretically in such a case a muscle graft would be preferable to an allograft and might be undertaken in preference to no treatment at all. Such cases are very limited in their incidence and alternative procedures such as intercostal nerve transfers and rerouting of tendons etc. reduces their number further. Ideally the muscle graft and cable graft need to be compared in a controlled clinical trial, this in turn following upon an experimental comparison of the two techniques. The present study, undertaken in sheep, involves the use of muscle grafts to repair the femoral nerve, which is larger than the nerves present in the animal models previously available to us. This nerve is of a similar size to many medium-sized and frequently injured human nerves containing the entire congeries of nerve fibre sizes (*e.g.* median, ulnar etc). The muscle graft is compared with a cable graft repair using a small expendable nerve as donor.

Materials and methods

Twenty-four young adult Suffolk–Dorset cross sheep of initial weight 30–40 kg were divided into two equal-sized experimental groups receiving, respectively, cable grafts or muscle grafts. Four sheep from each group were studied at periods of 4, 6 and 10 months after graft implantation. Initial preliminary studies of nerve fibre numbers in the sheep femoral nerve, saphenous nerve and lateral cutaneous nerve of the thigh had indicated that a cable composed of three strands of lateral cutaneous nerve would contain about the same number of myelinated fibres as the femoral nerve (see Results). Hence all cable grafts were made of three strands of lateral cutaneous nerve and the femoral nerve was used as the recipient site for both cable and muscle grafts.

The sheep femoral nerve was chosen as a relatively large mixed nerve (about the same size as the human ulnar nerve). Distally the only autonomous area relates to the saphenous branch, is very small and causes little, if any, functional loss with no damage to the hoof area consequent upon anaesthesia, allowing the sheep to return to a relatively normal life during the experimental period.

Each sheep was anaesthetised with halothane in 50% each of oxygen and nitrous oxide and intubated endotracheally for maintenance anaesthesia with spontaneous breathing. Pedal reflexes were monitored for depth of anaesthesia and the percentage of halothane adjusted to maintain appropriate levels.

With sterile surgical techniques, the right groin was prepared with Betadine in spirit and a horizontal incision made about 1 cm distal to the skin crease exposing the lateral cutaneous nerve. Where cable grafting was intended, 15 cm of lateral cutaneous nerve was excised, divided in three and preserved in a swab soaked in sterile saline. In animals receiving muscle grafts the sheep's rudimentary sartorius muscle was elevated from the underlying tissues over about 10 cm and divided at each end with the cutting diathermy. The harvested muscle graft was frozen to thermal equilibrium in liquid nitrogen and thawed in distilled water—during which time it underwent about 33% shrinkage—and fashioned into a rectangular block about 5 cm in length by 0.5 cm by 0.5 cm (Glasby *et al.*, 1986a, b, c, d; Glasby, 1990). For experimental conformity, the lateral cutaneous nerve was divided in those sheep receiving muscle grafts; likewise sartorius was excised in those sheep receiving cable grafts.

After removal of sartorius the recipient site was prepared by careful dissection of the femoral nerve off the lateral aspect of the femoral artery. The entire length of the nerve was isolated, from its emergence below the inguinal ligament down to the point at which it breaks up into a bundle of (mainly muscular) branches some 6–8 cm on. The nerve was cleanly divided using nerve-holding forceps and a razor blade fragment 1 cm distal to the inguinal ligament. Where the saphenous nerve originated proximal to this point, it too was divided. The muscle graft was interposed in the gap and secured by about six epineurial 10/0 polyamide monofilament sutures. The three-strand cable grafts were interposed using conventional funicular repair with 10/0 or 11/0 monofilament polyamide. In all cases a Wild M690 motorised x-y-zoom operating microscope (Wild-Leitz, Heerbrugg, Switzerland) was used and all operative procedures performed by the same individual (MAG). The muscle and soft tissue defects were repaired with 3/0 Vicryl (Ethicon); a similar suture was used for subcuticular approximation of the skin. Where necessary mild postoperative analgesia was given and the sheep were maintained in the animal house until ambulant (< 24 hours), and until the wound was healed. Thereafter they were returned to pasture with no restrictions on their activity.

At their allotted times the sheep were anaesthetised and the graft site exposed. A variable and inconsistent amount of fibrous tissue was encountered and dissected off the graft. After identification of the graft by the suture lines the femoral nerve was divided at the inguinal ligament and as far distally as was practicable. The normal femoral and lateral cutaneous nerves from the opposite leg were similarly removed to act as controls. The specimens were immediately immersed in 2.5% phosphate-buffered glutaraldehyde (pH 7.2–7.4) and fixed for a few minutes; this made it easier to cut transversely into proximal stump, graft and distal portions. It was then removed and the three parts corresponding to proximal nerve, graft and distal nerve were each sliced transversely in order to furnish small enough specimens for adequate fixation and osmication. Additionally this procedure ensures true transverse sections which are essential for morphometric analysis. These were returned to buffered glutaraldehyde for complete fixation (3 hours) and were subsequently immersed in 1% osmium tetroxide for 12 hours. Subsequently they were dehydrated, impregnated and embedded in Araldite and cut on an LKB 3 (LKB, Sweden) ultramicrotome to provide 1 μm (semithin) sections for light microscopy and ultrathin sections for electronmicroscopy. Sections for light microscopy were stained with toluidine blue and ultrathin sections for electronmicroscopy with lead citrate and uranyl acetate. For qualitative examination of the specimens the electron microscope was used to produce photomicrographs with a magnification of 3000 times. This gives superior resolution of detail to those obtained with the light microscope. For myelinated fibre counting and estimation of fibre size the semithin sections were adequate. Absolute total myelinated nerve fibre numbers were counted using a compound microscope (Jemamed, Carl Zeiss, Jena) connected to a Vids III semi-automated image analysis system (Analytical Measuring Systems Ltd., Pampisford, Cambridge). Distribution histograms of myelinated nerve fibre diameters were compiled from the same sections using a Magiscan M2A automated image analysis system (Joyce-Loebl Ltd, Gateshead, Cleveland, UK). Artefact rejection was set to eliminate fibres in the 0 to 0.75 range of the function $(4\,Pi.A)/p^2$, where Pi = 3.14286, A = cross-sectional area (microns^2) and p = perimeter (microns). This excludes nerve fibres not cut in transverse section and those showing excessive crenation due to poor fixation. Statistical comparisons of counts and fibre diameter measurements were made using Student's t test and exact values of "p" were obtained from the computer. The statement "p = 0.000" therefore indicates a value correct to 3 significant figures rather than an absolute value of zero, *i.e.* the significance of the calculated value of "t" is better than 99.999%. Fibre size distributions were compared using ogival (cumulated frequency) curves.

Results

Simple observation of the individual sheep showed very little functional deficit and remarkably little wasting of the knee extensors. At operation there was some scar tissue to be found in every case but more in relation to the repaired sartorius than the graft site. The latter was easily found in every case though in a few it had to be dissected free of the femoral artery. The cable grafts appeared to be associated with more fibrous and fatty tissue infiltration than the muscle grafts though no formal quantification was attempted. Cable grafts retained their three-stranded configuration up to 10 months; the gaps between the strands were filled with fat. Muscle grafts had remodelled as reported by Glasby *et al.* (1986a, b), with the appearance, colour and consistency of a thickened portion of nerve.

Figure 1 shows the mean numbers of myelinated nerve fibres calculated from absolute counts of transverse sections of femoral, lateral cutaneous and saphenous nerves. In each case N = 12 sheep. The mean values are femoral nerve = 8371 ± 116, lateral cutaneous nerve = 2972 ± 132 and saphenous nerve = 718 ± 14. From these values it was decided that a three strand cable made from the lateral cutaneous nerve would furnish approximately $3 \times 2972 = 8916$ endoneurial tubes which in theory should accommodate the 8371 fibres available from the femoral nerve. The larger number of saphenous nerve strands necessary would have made the surgery considerably more difficult. As far as one may make clinical comparisons this represents a generous graft.

Figure 2 shows the calculated means $\pm$ standard error of the mean of the total numbers of myelinated nerve fibres observed in the distal nerve beyond the muscle or cable graft in each of the experimental groups at 4, 6 and 10 months. There was a greater number of axons traversing the muscle graft at each time period, with the difference highly significant at 10 months ($p = 0.0023$).

Figure 3 indicates that although the number of fibres reaching the distal stump increased with

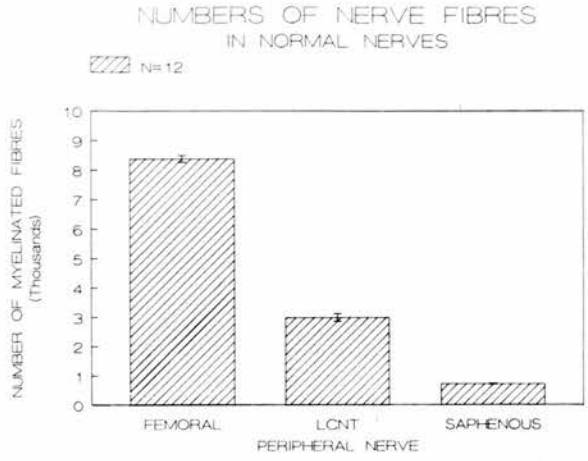

Fig. 1

Figure 1—Mean numbers of myelinated axons contained in the femoral nerve, the lateral cutaneous nerve of the thigh and the saphenous nerve of the sheep. In order to construct a cable graft, three strands of the lateral cutaneous nerve were used to supply approximately the same number of endoneurial tubes as were contained in the femoral nerve.

time, at 10 months it was still significantly lower than the number in the control (normal opposite leg) femoral nerve (8371 ± 116 compared to 5057 ± 207, $p = 0.000$ for a muscle graft and 3474 ± 218, $p = 0.000$ for a cable graft).

The level of maturation attained by the pioneering axons reaching the distal nerves was assessed at 10 months by measuring the diameters of the myelinated fibres and by comparing the distributions of fibre diameters in the two groups and in the controls. Figure 4 shows a comparison of the mean $\pm$ standard error of the mean diameter of control nerve fibres with similar observations from the two experimental groups. Each value represents the calculated mean of approximately 4000 observations pooled from the four sheep in each group. While there was no significant difference in mean diameter between fibres traversing cable and muscle grafts, both experimental groups contained fibres whose mean diameter was significantly smaller than that of the control nerves.

Figures 5, 6 and 7 are histograms showing the distribution of myelinated fibre sizes in normal femoral nerve, muscle graft and cable graft respectively. Each represents observations made on a single sheep with the corresponding mean displayed on the graph. The mean values plotted in Figure 4 were compiled from four such histograms which were also used to compile the ogival curves in Figure 8. These histograms show quite clearly the difference in fibre size distribution between control and experimental groups and the similarity of the two experimental groups.

Figure 8 is an ogival curve with each line representing the mean $\pm$ standard error of the mean of the cumulated percentage frequencies of fibre diameters from each of the four sheep in each group. The x-axis therefore corresponds to the

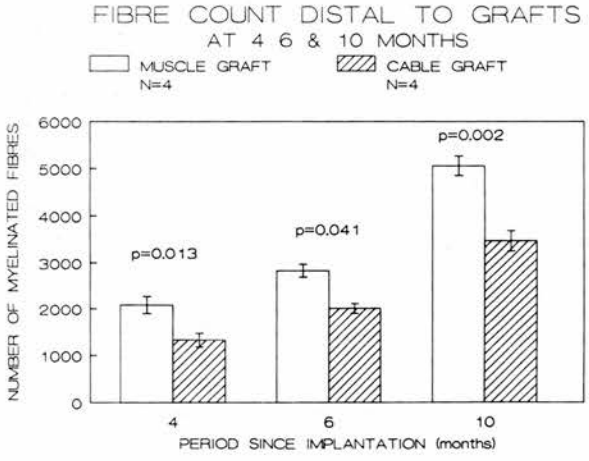

Fig. 2

Figure 2—Mean numbers of myelinated axons observed in the nerve segments distal to implanted freeze-thawed muscle and cable grafts at 4, 6 and 10 months after implantation. N = 4 sheep in each case.

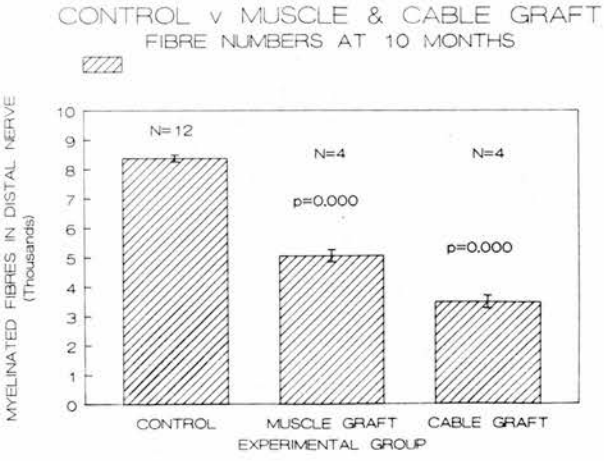

Fig. 3

Figure 3—Mean numbers of myelinated nerve fibres in nerve segments distal to muscle and cable grafts compared with numbers in the equivalent normal nerve 10 months after graft implantation.

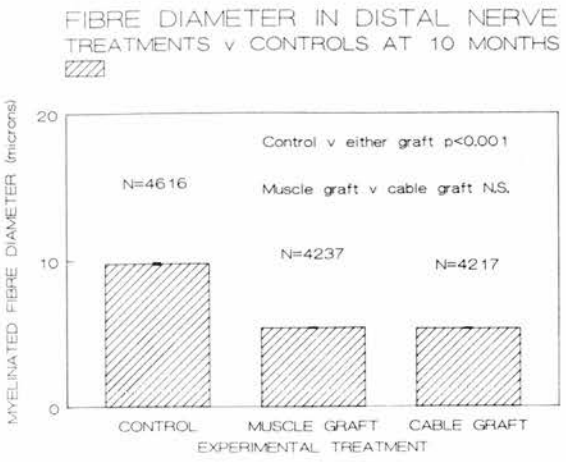

Fig. 4

Figure 4—A comparison of the mean diameters of myelinated nerve fibres in the normal femoral nerve with values obtained in nerve segments distal to muscle and cable grafts at 10 months. Both experimental groups are significantly different from controls but are not significantly different from each other.

range of bin sizes in the histograms from which the observations were taken and the y-axis to the mean cumulated frequency of the percentage of fibres in each bin. Each value thus represents the mean for the four sheep in each group. At any point on the curve, therefore, 'y%' of the total number of myelinated fibres would have a diameter not exceeding that of the corresponding value of 'x'. It can be seen that the control curve lies far to the right of the two sets of experimental observations. In the latter two groups, that corresponding to fibres traversing the muscle graft lies to the right of that of the cable graft for most of its length though the difference is unlikely to be significant. This is in agreement with the comparison of the mean values seen in Figure 4 and additionally shows the relationship to be consistent across the whole range of the distribution.

Figure 9 is an electronmicrograph of normal sheep femoral nerve corresponding to the controls used in the experiments. Large and small myelinated axons and their associated Schwann cells are clearly visible along with groups of unmyelinated fibres and small quantities of endoneurial collagen. This appearance is typical of mixed peripheral nerves in mammalian species.

Figure 10A is an electronmicrograph of a transverse section through the mid point of a muscle graft which had been inserted into the femoral nerve 10 months previously. Figure 10B is the corresponding segment of nerve distal to the graft. In the section of the graft there is a clear delineation of the axons into "minifascicles" each with a laminated surround of perineurial cells (Thomas and Jones, 1967; Morris *et al.*, 1972; Mackinnon *et al.*, 1985; Gschmeissner *et al.*, in press). Myelinated and unmyelinated axons are clearly visible though the absolute fibre size and the degree of myelination fall short of that in control nerves. The level of maturation of the nerve fibres in the distal stump corresponds to that in the graft but here there is no sign of the compartmentation seen in the graft. These figures should be compared with Figures 11A and B which are corresponding sections through a cable graft and its distal segment. Nerve fibre maturation looks very much the same but in the cable graft there is no compartmentation. In the cable graft there appears to be a more intimate relationship between the collagen and the neural elements, the latter being sequestered from contact with collagen by the perineurial cells of the minifascicles in the muscle graft. In both distal segments the distribution of collagen is as would be expected in any regenerating nerve.

Discussion

Freeze-thawed coaxial skeletal muscle autografts are considerably easier to harvest and insert than cutaneous nerves for cable grafts and they appear to function *at least as well* as conventional cable grafts. Specifically the results show that greater numbers of fibres pass through the muscle graft to reach its distal stump than through the cable graft by 10 months although at this time the pattern of regeneration and the maturation of the pioneering fibres is indistinguishable between the two groups. The essential difference lies in the morphology of the graft itself. Cable grafts show an arrangement of their contents indistinguishable from the distal stump of a nerve which has been transected and repaired. Muscle grafts show a strongly compartmented arrangement which persists in the short term (throughout the experimental period considered here or for at least a year in the rat). Neither of these configurations seems to affect regenerative capacity in terms of indices of maturation, though fibre numbers passing through muscle grafts are consistently greater. This might be no more than a reflection of its greater potential for forming minifascicles. The phenomenon of compartmentation has been extensively considered by Morris *et*

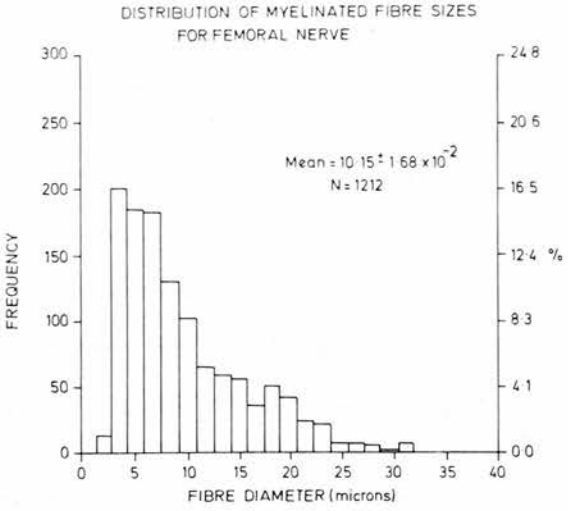

Fig. 5

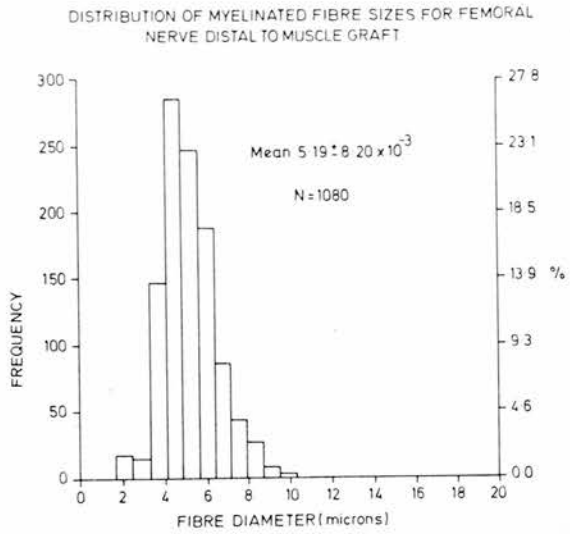

Fig. 6

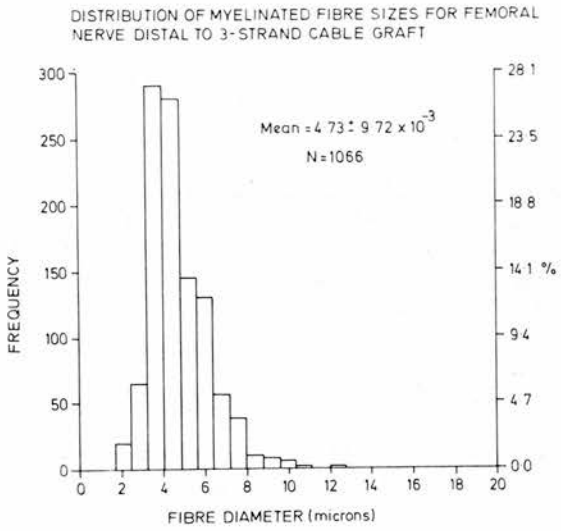

Fig. 7

Figure 5—Frequency histogram showing the distribution of myelinated fibre diameters in the normal sheep femoral nerve. **Figure 6**—Frequency histogram showing the distribution of myelinated fibre diameters in the nerve situated distal to a freeze-thawed muscle autograft implanted 10 months previously. **Figure 7**—Frequency histogram showing the distribution of myelinated fibre diameters in the nerve situated distal to a 3-strand lateral cutaneous nerve autograft (cable graft) implanted 10 months previously.

al. (1972), who viewed the process as being one in which the presence of axons in relation to non-neural tissue causes a reorganisation of the latter such that, "while the survival of the axons is ensured by the development of compartmentation, their regeneration is impaired relative to their maximum capacity". Compartmentation is thus the anatomical expression of a mechanism designed to re-establish the physical coverings of the funiculus and thereby restore the endoneurial environment. The minifascicle is enclosed within a laminated perineurial cell surround which provides a diffusion barrier between the extracellular and the endoneurial spaces (Huxley and Stampfli, 1951; Shanthav-

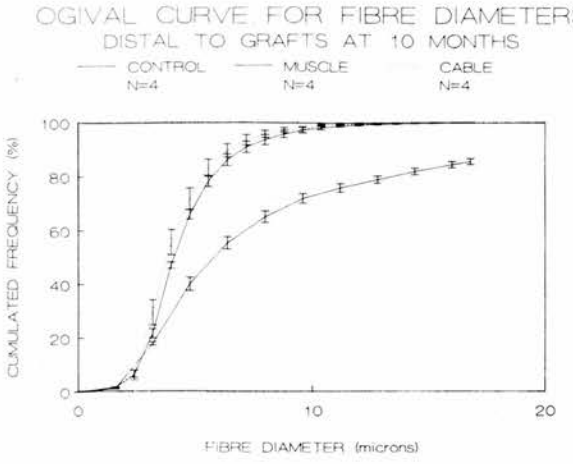

Fig. 8

Figure 8—Ogival curve showing cumulated frequencies of distribution of mean myelinated fibre diameters from Figures 5–7. In each case the data points represent the mean observations from 4 sheep. See text for explanation.

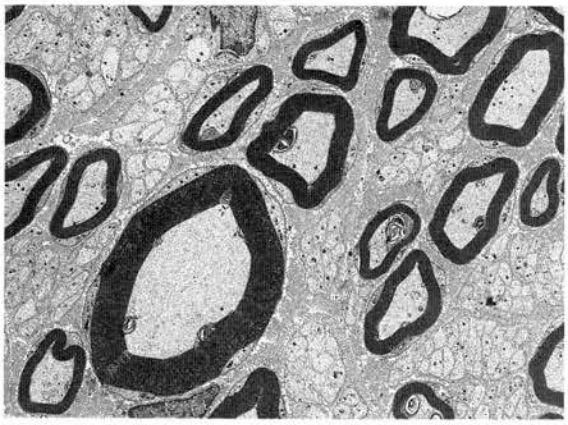

Fig. 9

Figure 9—An electronmicrograph (×1380) of a transverse section of the normal sheep femoral nerve at the level of grafting. Note the presence of both mylinated and unmyelinated nerve fibres.

eerappa and Bourne, 1962, 1963, 1968). In the rat, where a nerve is repaired by primary suture, minifascicles reach peak numbers by 26 days and thereafter coalesce to produce an arrangement of fascicles not unlike those of the parent nerve (Morris *et al.*, 1972; Mackinnon and Dellon, 1988; Gschmeissner *et al.*, in press). In the case of muscle grafts, although this process occurs in the distal nerve stump the minifascicles remain in the graft. It would be wrong, however, to suppose that the recovery of larger fascicles in a direct repair or following insertion of a cable graft necessarily implies restoration of the original architecture. The process of compartmentation itself begins in the proximal stump in all cases (Morris *et al.*, 1972), where it must be associated with considerable disorganisation of the original "wiring pattern", and the results of these workers must call into question the entrenched surgical philosophy that the coaptation of endoneurial tubes is the *sine qua non* of effective nerve repair (Glasby, 1990). Morris *et al.* (1972) suggest that there are two cardinal principles to be followed in nerve repair: (1) that the separation of nervous tissue from mesodermally-derived tissue must be maximised and (2) that preservation and reconstitution of the endoneurial environment must occur. The natural process aimed at both of these ends is compartmentation and the cost is the ineluctable forfeiture of precise rewiring. The extent to which variation of the surgical technique can contribute to these two aims is debatable. In theory at least, funicular repair should be a nonsense but this is clearly not so, at least where the fascicles are large and few in number. Presumably although compartmentation must occur within the fascicle, axon types remain grouped together in roughly constant proportion and geometrical relationship to one another and hence benefit accrues if it is attached to a similarly composed and orientated fascicle. If one accepts the phenomenon of compartmentation, however, orientation can only be of value at the perineurial level and certainly not at the level of the endoneurial tube and funicular repair only makes sense where a single funiculus with its external perineurial lamina is joined to a funiculus of like composition. Had the work of Morris *et al.* (1972) been published in a journal more accessible to the practising surgeon, the development of nerve repair over the last twenty years might have been significantly different.

In the relatively small number of cases in which muscle grafts have been used clinically to repair sensory nerves (Norris *et al.*, 1988), they have been associated with a very marked and welcome absence of hyperaesthesia. This, like the pain of a neuroma, appears to occur when a disorientated array of regenerating axons is associated with an excess of ectopic connective tissue. In all of the experimental muscle grafts which have been retrieved it has been observed that the proportion of connective tissue to neural tissue is remarkably small. Formal

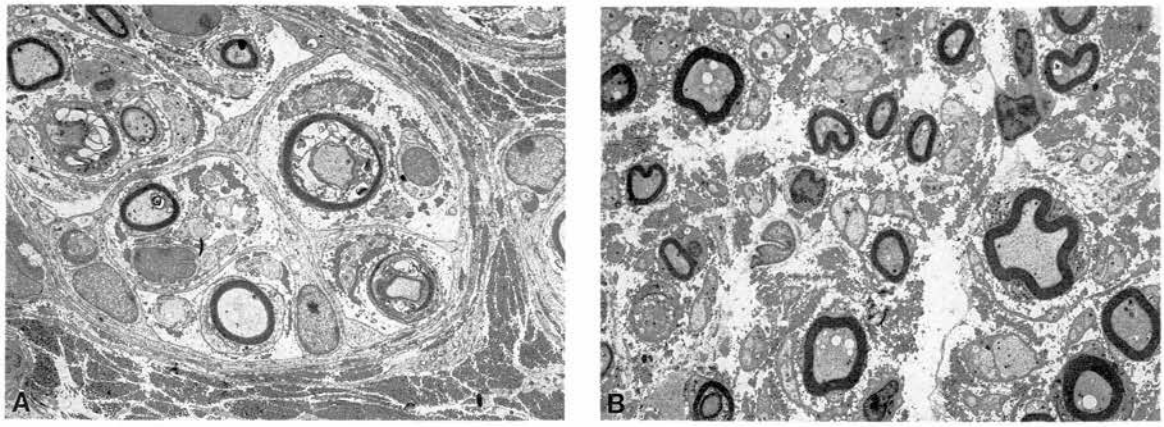

Fig. 10

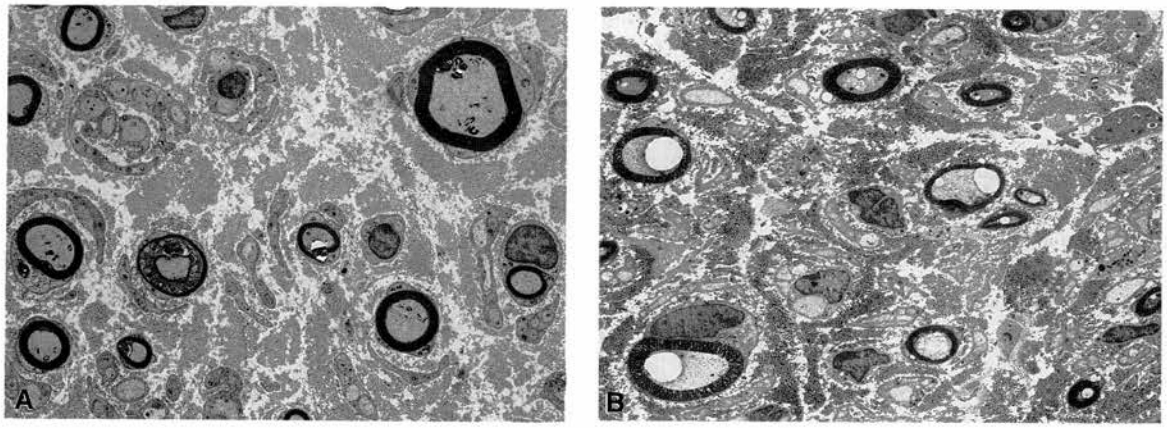

Fig. 11

Figure 10—(A) An electronmicrograph (× 1380) of a transverse section through a freeze-thawed muscle graft implanted 10 months previously. Note the marked compartmentation present throughout the graft and the sparse arrangement of the endoneurial collagen. (B) An equivalent section through the corresponding distal nerve stump where compartmentation has resolved. **Figure 11**—(A) An electronmicrgraph (× 1380) of a transverse section through a three strand cable graft implanted 10 months previously. Note the lack of compartmentation in the graft and the abundant collagen. (B) An equivalent section through the corresponding distal nerve stump.

quantification of this is currently in progress in our laboratory. In contrast, the cable graft appears to acquire the maximum connective tissue infiltration seen in any form of nerve repair except for those using foreign or artificial substances. The electronmicrographs shown here suggest that it is not the absolute amounts of collagen which may have an effect but rather the spatial distribution inside and outside the compartmented perineurial cell sheaths. In the muscle graft there is very little collagen to be seen within the perineurial sheath whereas in the cable graft each axon is exposed to a greater and more uniform distribution of collagen. Thomas and Jones (1967) have differentiated endoneurial collagen with its thinner diameter of 51.5 nm from typical mesodermally-derived collagen (80 nm), and Morris *et al.* (1972) have suggested an ectodermal origin for the fibroblasts producing the former along with a possible three-way transformation between these cells, Schwann cells and perineurial cells. It is not clear from the present sudy whether this is the case.

While the muscle graft increases the prospect of more axons passing through, it may be the case that

myelination within the graft is more sparse than in the corresponding distal nerve (Glasby *et al.*, 1986c, d). This non-uniformity, which may be a consequence of restricted Schwann cell entry, should be associated with a non-uniform conduction velocity. However, this does not produce any measurable functional defecit (Glasby *et al.*, 1988a).

In conclusion, the muscle graft used in the sheep femoral nerve represents a technically more simple method of repairing a large mixed nerve than inserting a three-strand cable graft. At all times after graft insertion, a greater number of axons had regenerated through the muscle graft than through the cable graft and at none of the times studied were the indices of fibre maturation and, by limited extension, function, significantly different. It seems likely that muscle grafts could be used successfully for the repair of large mixed nerves injured in a manner currently best repaired with a cable graft. The present results may thus be of value in a consideration of the ethics of such a step in clinical practice.

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